

***DROSOPHILA MELANOGASTER* OVIPOSITION AND
TOXICITY STUDIES ON A CHIP**

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ABSTRACT

Oviposition or egg-laying is an important behaviour used to assess the biological processes in *Drosophila melanogaster*. This behaviour is affected by physical and chemical properties of the substrate, which have not been investigated precisely and parametrically with existing manual approaches. Current oviposition-based chemical screening studies using agar plates are inaccurate, labor-intensive, and inflexible due to the manual chemical doping of agar. In this thesis, we have devised a miniaturization method to precisely and repeatedly manipulate agar stiffness and exposure area to quantitatively study their effects on oviposition in *Drosophila*. Using this method, we have also developed agar-polydimethylsiloxane microfluidic devices for single- and multi-concentration chemical (zinc and acetic acid) dosing and on-chip oviposition screening of *Drosophila*. A 1% exposure area was found to provide pure agar-like oviposition, and our microfluidic devices demonstrated chemical concentration dependency in oviposition responses. These devices may be further used for assaying fundamental oviposition questions, learning, and decision-making phenomena in *Drosophila* and other egg-laying insects.

DEDICATION

To my Dearest Family, Friends, and BrOTs.

-JACOB 

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From the bottom of my heart, my deepest gratitude goes to everyone that has help made this possible. Without your help and support, I would not have made it thus far. First and foremost, I would like to sincerely acknowledge my supervisor, Dr. Pouya Rezai, for taking me on as his first graduate student, and giving me the freedom and opportunity to learn and be creative in his laboratory. Without his value for creativity, leadership, time, guidance, and understanding, I would not have been able to succeed and accomplish so many things that quickly within these two years of my graduate studies in mechanical engineering.

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Soli Deo Gloria

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Chapter 1

Introduction

Drosophila melanogaster, commonly known as the fruit fly, is one of the model organisms that is most widely used to study human diseases, which has contributed significantly to various areas of neurobiology, genetics, developmental studies, and toxicological sciences [1–6]. This is mainly due to its rapid reproduction and development, small size, ease of caring and genetic manipulation [7], and its genetic homology to humans [6] in many known and conserved genomic sequences [3,8], which are used to investigate the root causes and the mechanisms behind human diseases and disorders down to a molecular level [3,8–11]. Thereby, chemical screening on *Drosophila* is of central importance in applications such as drug screening and toxicity studies. Toxicity is defined as the dose-dependent effect of chemicals on the overall animal in the areas of viability rate, delays in development, fecundity, or the changes in the physical appearance and chemical resistance in the body due to the environment [12]. Thus, *Drosophila* is also frequently used in ecological [10,13,14] and agricultural [15,16] studies as a “test-insect” [17–19] whereby genetic and metabolic pathways or behavioural responses of *Drosophila* are assayed. *Drosophila* is generally used to observe insecticidal as well as insect attraction and repellence activities towards certain toxins or chemical compounds in a substrate. In these studies, behaviours such as oviposition [20],

movement [21], foraging [22], avoidance [23], and mating [24] are assayed in response to environmental toxicants [1,24–26].

1.1 Importance of Oviposition in *Drosophila melanogaster*

Oviposition is the process of egg-laying in *D. melanogaster* that is dependent on responses of the fly to environmental stimuli (such as chemicals, physical conditions of the substrate, temperature, light, population density, and humidity) [13,27–30]. *D. melanogaster* possesses sensory neurons (such as taste [31,32], touch [33,34], and olfactory [15,35] sensing systems) to detect various environmental cues prior to exhibiting avoidance or selecting preferred sites for egg laying [13,27–30]. Therefore, oviposition (in conjunction with viability of the adult fruit fly) is a metric quantity that provides a clear indication of flies' sensory system health and their overall biological fitness (the ability to survive and reproduce) [13,27–30,35,36]. It is used predominately in developmental [18], chemical screening [37], and toxicology [38,39] studies as a simple and effective readout indicator. It is also of interest to biologists for answering fundamental questions about oviposition and reproduction patterns in insects. Thereby, development of a precise and sensitive method for accurate and quantitative analysis of oviposition is necessary.

1.2 Oviposition Studies in *D. melanogaster*

Current ovipositional assays in laboratory settings investigate the effects of environmental cues such as physical surface texture [40,41] and substrate chemical composition [36] on oviposition by using the standard yeast medium and the grape juice agar plates [42]. Ovipositional studies may be divided into two main categories: physical texturing topology and chemical substrate composition studies.

1.2.1 Effects of Substrate Physical Properties on Oviposition

Current surface texture studies may be further classified into additive and subtractive texturing approaches. Former methods have used seeded bisected grapes [41] or blotters [43], silk meshes [40], and different kinds of papers [40] (such as cardboard strips, filter paper, and Washi paper) on top of standard agar substrates to produce different surface modifications. Similarly, to produce texturized surfaces, manually scratched agar plates [43,44] were used in the latter approaches. In a study conducted by Ruiz-Dubreuil et al., grapes (seedless and seeded) were bisected manually to expose the inner flesh and were used as the control and textured substrates in the assay respectively [41]. Then, flies were transferred into vials containing either the top or the bottom half of the bisected grape and left to oviposit for an hour [41]. From this experiment, Ruiz-Dubreuil showed that *D. Melanogaster* oviposited more on seeded grapes than on the seedless for both the top and the bottom half of the bisected grapes. Although this study has shown an association between relatively smooth and surface modified substrates, it did not consider the differences in shape, size, and taste of the different kinds of grapes.

Also, the amount of seed in each seeded grape was not quantified or measured, which produces variations in the degree of roughness. Likewise, Takamura and Fuyama also conducted experiments on the effect of surface texture on fly oviposition using pre-existing objects. In the first two experiments, a strip of cardboard (1mm thick), filter paper (0.5mm thick), thin Japanese paper (0.02mm thick), and silk screen (180 μ m thick) were vertically erected or horizontally placed directed on the standard yeast medium [40]. Then, 15 female flies were allowed to oviposit in the dark, and the amount of eggs that was laid on the media and on the strips were counted. From this, flies showed a significant preference to all paper materials due to their textures. Moreover, they investigated the tendency for *Drosophila* to oviposit and insert their eggs into the medium. Different silk meshes (76.1 μ m, 92.1 μ m, 120.5 μ m, 155 μ m, and 210.1 μ m) were used to cover half of the standard medium. From this, they concluded that flies require the hole-sizes in the mesh to be greater than 155 μ m in order to be able to discriminate the texture induced for oviposition. Via using the additive texturing methodologies, more eggs were shown to be laid on certain substrates modified either by silk meshes or seeded grapes [40,41]. However, due to the low mechanical stiffness of the silk meshes, their physical shapes were reported to be deformed upon implantation of the eggs [40].

Subtractive texturing methods have also provided the same conclusion that textured substrates induce more oviposition. It was shown that egg-laying was enhanced and more prominent on rough surfaces and on sharp edges produced by manual incisions of substrates with sharp objects (as seen in Figure 1a) to create slices, slits, and grooves

[29,43–45]. Atkinson showed that texture is important in oviposition when investigating the reason for gregarious ovipositional effects [45]. They created 12 egg-laying pots filled with yeast-agar and patterned half of them manually by scratching a 5mm deep perpendicular grid with a sharp needle (Figure 1b). It was observed that 90.6% of the eggs were laid in these conditioned chambers.

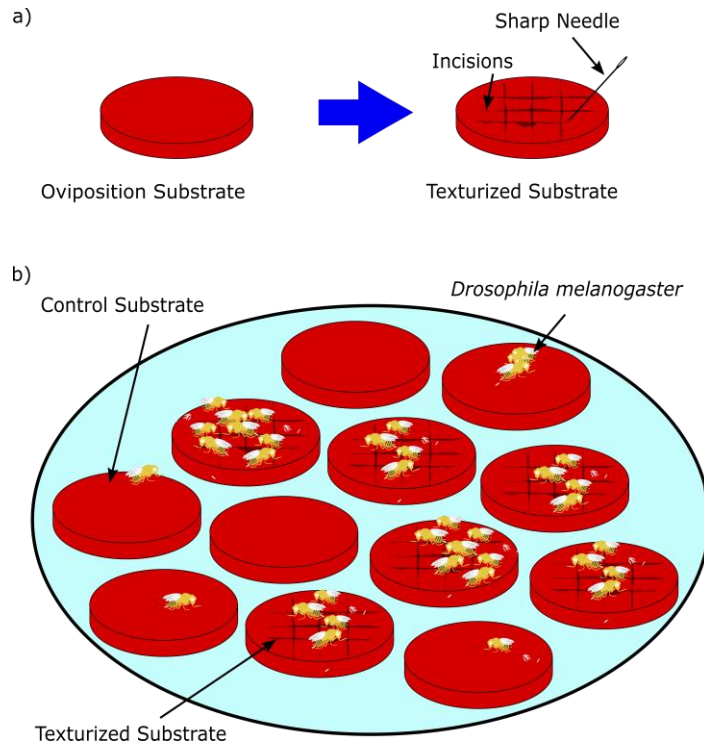


Figure 1: (a) Manual incision technique and (b) oviposition assay on surface textures conducted by Atkinson[45]. As seen in (a), current physical topology studies manually scratch the soft oviposition agar substrate with a sharp object (*e.g.* a needle). By doing so for six substrates and assembling them on a plate with six corresponding control (un-textured) substrates (as in b), adult flies were allowed to choose their preferred oviposition site. Atkinson observed significant amount of oviposition on the textured substrates in comparison to the controls.

1.2.2 Effect of Chemicals on Oviposition

Aside from the manual techniques that were used to create physical properties on the substrates, chemical properties were also shown to influence the adult flies' oviposition significantly. Current studies have shown that various toxins and chemicals (at different concentrations) induce ovipositional behaviours in *D. melanogaster*. In many of such studies, the oviposition platforms used in the laboratory were the conventional grape juice agar plates [42] and the standard yeast/cornmeal medium [39]. Chemicals at different concentrations were mixed into these media substrates, and the solidified agars were manually cut, removed, and reassembled onto a petri dish for oviposition experiments. Using this method and in a toxicity study by Bahadorani and Hilliker, ovipositional responses of *Drosophila* were shown to be greatly influenced by heavy metals such as cadmium, copper, iron, and zinc at different concentrations [36]. Specifically for cadmium and zinc, it was shown that adult flies preferred to oviposit on low concentrations (4mM and 2mM, respectively) while avoiding high concentrations (20mM and 70mM, respectively) of chemicals. In another study, Joseph *et al.* showed that an agar substrate doped with 5% acetic acid renders it highly attractive to *Drosophila* for oviposition [35]. Other studies have also shown oviposition attraction towards chemicals such as limonene [46] and octanoic acid [37], but demonstrated avoidance to lethal ones such as geosmin [15], using similar manual oviposition substrate preparation methods [35,47]. Although the agar doping approach has demonstrated promising results for screening of toxins and chemicals on oviposition, the assay platforms bear a number

of disadvantages, such as being time-consuming and labor-intensive to prepare, having large footprints due to the oversized dimensions of the oviposition sites, being amenable to chemical evaporation and change of concentration during prolonged oviposition assays and lacking the ability to control chemical exposures during the experiments. Because the solid agar pieces have to be premade and assembled in the dish, the interface boundaries between them can generate physical topographies on the substrate that are highly attractive as a site for oviposition [35] (as seen in Figure 2), causing a lack of repeatability and negatively affecting the accuracy and precision of the chemical assay. Moreover, to date, many studies have only used single concentration settings (low throughput setups) to determine if a chemical is an attractant or a deterrent reagent, hence giving less attention to high throughput investigation of the effect of chemicals and their concentration on oviposition. Accordingly, there is a need for development of a technology with higher throughput and controllability for assessment of oviposition of fruit flies in response to single- and multi-concentration exposures to toxic and attractive chemicals. This technology must be amenable to parallelization in order to be able to increase the throughput of the assays to 10s and possibly 100s of chemicals at a time in the future.

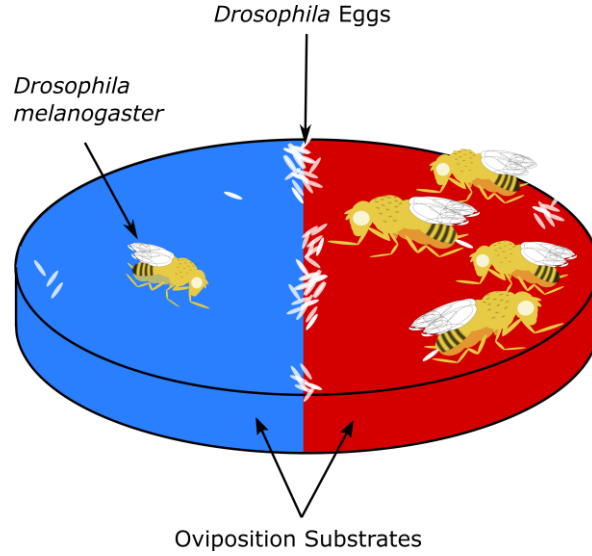


Figure 2: Conventional experimental setup for assaying *Drosophila* oviposition behaviours in response to chemical properties using the off-chip dosing method. In this method [35], the first assay agar medium was prepared and cured onto a dish and was manually cut into half. One half of the agar (*i.e.* red) was removed while another doped liquid agar was prepared off the oviposition platform. This doped liquid agar (*i.e.* blue) was then poured and cured into the void.

1.3 Micro-technology in Current *Drosophila* Research

Recently, microfluidics and micro-electrical-mechanical systems (MEMS) have been successfully used to enhance accuracy and reproducibility in many *Drosophila* assays [48–53]. The current MEMS and microfluidic chips used for fruit fly studies may be categorized into two main groups: (*i*) embryonic and (*ii*) larval study devices. For embryonic studies, Levario *et al.* microfabricated a PDMS device with precise microchambers arrayed on the side wall of a main channel, and immobilized embryos,

via manipulation of the flow and the pressure, for anterior and posterior imaging of the eggs in a high throughput manner (as seen in Figure 3a) [48]. Accurate and precise microneedle-based transgenic injection devices were also developed by Zappe *et al.* [49] and Delubac *et al.* [51] for single egg immobilization and microinjection with high success rates (*e.g.* 87% [51]) and automation levels [50,51]. Moreover, another embryonic chip with self-assembled oil covered thiol gold pads was designed by Dagani *et al.* to immobilize and to observe the cellularization of fly eggs when two fluids of different temperatures were laminarly flowed across their anterior and posterior segments [52]. From this, it was observed that a binary temperature produced asynchrony in the development of the membrane furrows with the warmer side producing a faster developmental rate in reference to the cooler side.

Similarly for larval studies, microfluidic chips have been designed for *in-vivo* imaging and quantification of neural and cellular responses of *Drosophila* larvae to various external stimuli [11,53,54]. Ghannad-Rezaie *et al.* developed a microfluidic chip to immobilize fluorescently tagged *Drosophila* larvae by a deflectable membrane and CO₂ exposure for long periods of time during imaging of neuronal activities (*e.g.* IV neurons) under a confocal microscope [53]. Other immobilization microfluidic chips were developed to observe vesicle transport in neurons and neuroblast developments [54]. Recently, Ghaemi *et al.* also developed two microfluidic devices for immobilizing the larva and live fluorescence imaging of the central nervous system activities in response to acoustic signals via the G-CaMP5 calcium sensor (Figure 3b) [11]. All these

miniature devices have shown great capabilities and high accuracies in quantitative and high throughput selection, orientation, immobilization, microinjection, and developmental studies of *Drosophila* embryos or larvae under controlled conditions. Thus, micro-technology is a promising tool for the investigation of flies at their different developmental life stages.

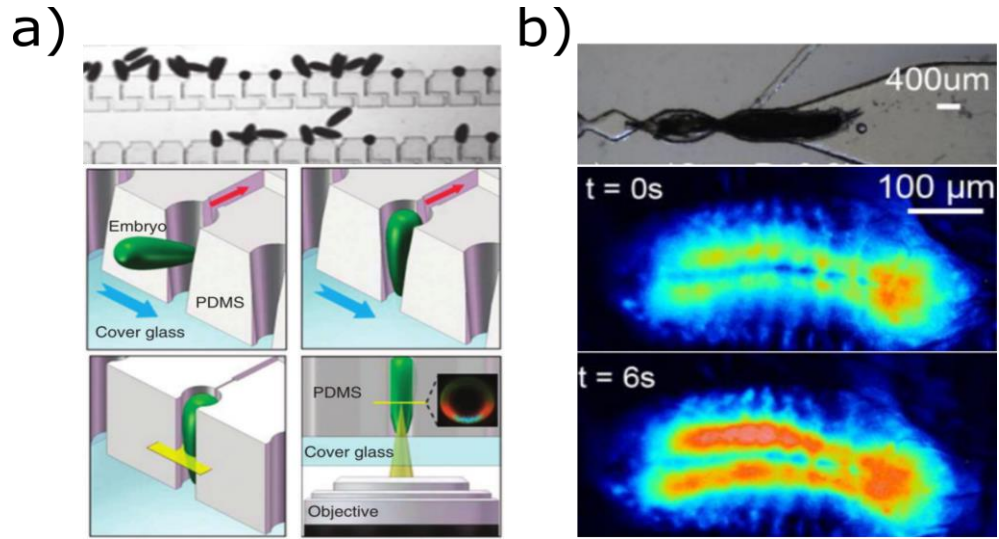


Figure 3: (a) Embryonic [48] and (b) larval [11] microfluidic chip for accurate imaging of development and neuronal activities. (a) Embryonic chip on the top with channels covered with traps as seen in the illustration below. As the embryos were passed through the main channel, the slits opened up, causing the embryo to be entrapped and immobilized in the anterior-posterior orientation for accurate fluorescent imaging. (b) Larval chip with the interior segment of the larva trapped pneumatically. This allowed *Ghaemi et al.* to immobilize the larva for accurate fluorescent imaging of the central nervous system provided with no stimuli (middle) and with acoustic stimuli (bottom). Reproduced with permission from [48] and [11].

1.4 Research Goals and Objectives

The main objectives of this research were to design a reliable tool for studying viability and oviposition behaviours quantitatively (with respect to texture and chemistry), and microfluidics devices for controllable and accurate on-chip chemical dosing and screening of egg-laying response of free-flying adult *Drosophila melanogaster* in the presences of single or multiple chemicals. To achieve this goal, the research was divided into three major objectives:

- (1) To determine if polydimethylsiloxane (PDMS), which is commonly used for developing microfluidic devices in fruit fly studies, can sustain the full viability rate in adult-staged *Drosophila*.
- (2) To develop a novel and sensitive tool (*i.e.* for miniaturization and precise manipulation of oviposition substrates) for studying the effects of substrate parameters (*e.g.* exposure area and inter-spacing) on adult fruit flies' viability and oviposition behaviour (collectively and at a single egg level) in a quantitative and convenient manner.
- (3) To innovate a process to integrate the miniaturized oviposition assay tool with a microfluidic module for controllable and on-chip single- and multi-chemical dosing of flies and toxicity studies based on oviposition.

Firstly, patterned PDMS through-hole membranes overlaid on conventional agar substrates were developed to perform highly controllable viability and oviposition studies on adult flies. This novel technique was proven to be a sensitive and quantitative tool for behavioural assays on *Drosophila melanogaster*. It may also be used for fundamental behavioural and genetic studies in *Diptera* (e.g. *Ceratitis capitata*) and other flying insects (e.g. moths) in the future. Integrating the PDMS-agar substrates into PDMS microfluidic platforms, two devices (viz. toxicity assay and multi-choice assay chips) were developed via an unprecedented ice sacrificial layer fabrication methodology. This microfabrication methodology is a cost-effective way to produce sacrificial layers in microfluidic devices by using the phase-change behaviour of water. The originality of this work is on the development of the sacrificial technique to integrate agar and PDMS-based microfluidic channels into a device, as well as the application of the developed devices for studying adult *Drosophila*'s mating and reproduction in response to chemicals at various concentrations. Our on-chip dosing and chemical screening devices may be further used for applications in the development of devices for decision-making and toxicological chemical screening assays or as tools for collection, de-clustering and self-assembly of eggs for embryonic imaging and developmental studies.

Chapter 2

Materials and Methods

Oviposition in adult flies is highly dependent on environmental stimuli, such as substrate topography and chemistry [55]. To investigate the effects of the physical characteristics of the substrate (*e.g.* agar exposure size, inter-spacing, and stiffness) on oviposition, patterned PDMS through-hole membranes overlaid on conventional agar substrates were used. These investigations provided fundamental understanding of *Drosophila's* oviposition site selection for designing the two novel chemical screening microfluidic devices: one for toxicity assays and the other for multi-choice preference chemical screening applications. This chapter details the preparation of the agar substrates, reagents, and the animals used followed by procedures to conduct the experiments with the sensitive agar-PDMS miniaturization and quantification tool as well as the two microfluidic devices.

2.1 Fly Stock

White (w^1) *Drosophilae* were grown on standard yeast media in stock bottles and maintained under standard 12:12hr light:dark lighting regime. For all experimental assays, adult flies were emptied from the bottles on the day of collection. Young flies that emerged from their pupae within half a day were collected into new stock bottles and were aged 5 days. To ensure an adequate amount of food source and constant population

density of flies, 0.3g of flies were anesthetized with ether and put into each of these bottles at 21 °C. When transferring 25 female and 20 male flies from the stock bottles to the assay set-up, flies were first anesthetized and counted. Prior to each assay, the flies were given a minimum of 1 hour recovery time.

2.2 Agar Preparation

Conventional agar-juice [42], a common laboratory substrate in oviposition assays, was used as the positive control substrate in the fundamental oviposition studies and in the toxicity and the multiple-choice assays using the microfluidic devices. This solution (10g agar, 11g sugar, 333ml distilled water, and 111ml Welch's frozen grape juice concentrate) was prepared via mixing at 700rpm and 200°C on a magnetic stirring hotplate until the solution was fully boiled. Subsequently, the solution was cooled at room temperature to 80°C by natural convection, and the bubbles were allowed to escape from the surface. Then, 3ml of the solution was plated onto a petri dish lid or inside a designated void (40mm diameter) on microfluidic devices using a syringe and left for solidification. The plated solidified agar substrates were used as controls and base substrates in the oviposition experiments discussed below. Whenever needed, PDMS membranes incised with through-holes were overlaid on top of the cured agar to control the amount of agar exposure to the adult flies.

2.3 Reagent Preparation

Methylene blue solution (4mg/mL) was used as a surrogate [56] for the chemical reagents used in the chemical screening assays. This solution was prepared by dissolving methylene blue powder into distilled water. In addition, two chemicals, zinc and acetic acid, were prepared at different concentrations and used in both the toxicity and the multiple-choice oviposition assays. The zinc solutions of three different concentrations (2mM, 20mM, and 70mM) were prepared by dissolving zinc chloride powder in double distilled water. Likewise, the three different acetic acid concentration solutions (1%, 5%, and 15%) were prepared by mixing glacial acetic acid and double distilled water. These solutions were then inputted into the designed microfluidic devices via the chemical containers as described in detail in the experimental assay section.

2.4 Experimental Setup for Oviposition Studies

The experimental setup for the fundamental oviposition assays (Figure 4) consisted of a stock bottle with air holes on the utmost top, containing $n=45$ flies (25 females and 20 males), assembled onto a patterned oviposition substrate (*i.e.* agar surfaces patterned with through-hole PDMS membranes as discussed in *Section 2.5*) and a dark cage for conducting the experiments. Using a small paint brush, the counted anesthetised flies were brushed into each stock bottle. Subsequently, the patterned oviposition substrate was capped onto the mouth of the stock bottle via a press fit. To

ensure the system was well-assembled, a piece of tape was used to secure the substrate to the bottle prior to the inversion to allow fly-substrate contact as in Figure 4. The whole assembly was then transferred into a dark and enclosed box, and the flies were left to oviposit for 24 hours at 21 °C. The start and end times for all the assays were the same to enhance the precision in the oviposition rate achieved and to remove the possibility and effects of clock gene dependency [57,58]. After the assays, the viability rate of the adult flies (*i.e.* the percentage of alive animals) and the ovipositional rates (*i.e.* the number of eggs oviposited inside and outside of the holes of the PDMS membrane) were quantified with the aid of a dissection probe and a dissection microscope.

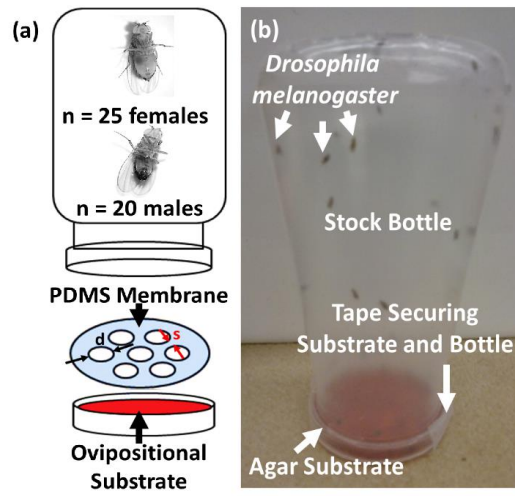


Figure 4: Schematic of the experimental set-up for ovipositional assay shown in (a) and the corresponding assembly shown in (b). The set-up consisted of 25 female and 20 male flies in an inverted stock bottle capped onto an oviposition substrate. The substrate consisted of a custom-patterned through-hole PDMS membrane that was overlaid on each agar juice plate. Various patterning configurations such as single through-holes (diameter $d=0.5, 2, 4$, and 8mm) and seven hexagonally-patterned through-holes ($d=0.5, 2$, and 4mm) with equal spacing ($s=0.5, 2$, and 4mm) were used for the assays.

2.5 Substrates in Oviposition Studies

The agar juice plates discussed in *Section 2.2* were used as positive controls and base substrates in the oviposition experiments with PDMS through-hole membranes overlaid on top of them for precise patterning. These membranes were made via mixing Sylgard 184 elastomer and curing agent in a 10:1 ratio (Dow Corning Co., Midland, MI, USA). Agar sugar content and stiffness properties were also altered in our assays. To produce negative control substrates (with no access to agar), 3ml of PDMS was plated onto the same petri dishes via a syringe and left to cure for a day at 21°C.

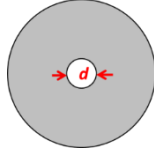
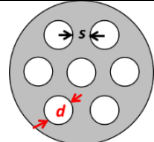
To vary the concentration of sugar and to study the effect of sucrose content of the agar-juice plates on oviposition, the amount of sugar was altered from the control recipe, which originally had 11g of sugar. The high- and low-sugar substrates in these assays contained 22g and 5.5g of sugar respectively with all other components kept unaltered as discussed above.

The effect of substrate stiffness was assayed by modifying the amount of agarose used in the conventional agar-juice plate recipe that contained 10g agarose powder. The soft and stiff agar substrates contained 5g and 20g agarose powder respectively.

Patterned PDMS through-hole membranes ($250\pm 50\mu\text{m}$ thick) were used on top of the abovementioned agar juice base substrates (Figure 4) to study the effect of surface area exposure on the survival and oviposition behaviour of fruit flies. To fabricate the PDMS membranes, 0.5ml of PDMS pre-polymer (Sylgard 184 elastomer and curing agent, mixed in a 10 to 1 ratio, respectively) was spun on a 6cm×6cm transparency film

using a spin coater (MODEL P6700 Series, Speciality Coating Systems Inc., Indianapolis, IN, USA) for 20 seconds at 600rpm. The pre-polymer was allowed to set and cure for 1 day at room temperature. To pattern the PDMS membranes, a single circular through-hole (diameter $d=0.5\text{mm}$, 2mm , 4mm , and 8mm) was punched into the fabricated membranes using the corresponding Harris Uni-Core™ hole-puncher (Ted Pella Inc., Redding, CA, USA). Each of these hole sizes correspond to the percentage exposure areas, as summarized in Table 1, defined as (area of through-hole/area of agar substrate) $\times 100\%$. On the other hand, multiple-hole membranes were produced similarly with the additional aid of a 3D printed incision guide. For assaying exposure hole diameter and interspacing, nine 3D printed molds, each containing seven-hole guides with diameters of 0.5mm , 2mm or 4mm and equal spacing of 0.5mm , 2mm or 4mm , were used to pattern corresponding through-holes hexagonally in PDMS membranes. After preparation of the desired PDMS membranes discussed above, they were lifted off their fabrication substrates and placed on the standard agar juice plates for oviposition and survival assays as shown in Figure 4 and discussed in *Section 2.4*.

Table 1: PDMS membranes used to pattern oviposition substrates

Number of Through-holes	Through-hole Diameter, d (mm)	Interspacing, s (mm)	<i>Exposure Area (%)</i>	Schematic
<i>Single Through-hole</i>				
1	0.5	-	0.016%	
1	2	-	0.246%	
1	4	-	1%	
1	8	-	4%	
<i>Seven Hexagonally Patterned Through-holes</i>				
7	0.5	0.5, 2, 4	0.112%	
7	2	0.5, 2, 4	1.75%	
7	4	0.5, 2, 4	7%	

The effects of patterning of the pure agar and PDMS substrates on oviposition were also investigated. For this purpose, two additional homogeneous substrates were fabricated: (i) a pure PDMS and (ii) a pure agar substrate. Both substrates contained a 4mm diameter and 0.25mm deep circular cavity in the center. They were called *conditioned PDMS* and *conditioned agar* substrates in our assays. To fabricate the *conditioned PDMS* substrate, a PDMS membrane with a punched 4mm-diameter hole was overlaid on a flat PDMS substrate. To produce the *conditioned agar* substrate, a 4mm PDMS disc was placed on top of a solidified agar-juice plate. Liquid agar-juice medium was then quickly transferred via a cotton applicator to create a uniform surface by filling up the area around the 4mm PDMS disc. Upon curing of the agar substrate, the PDMS disc was removed via a set of forceps to expose the agar cavity. These substrates

were also tested for oviposition as discussed in *Section 2.4*. Table 2 summarizes all parameters that were studied via the miniaturized oviposition substrates.

Table 2: Summary table of all the parameters investigated on oviposition

Parameters Studied on Oviposition	Details and Specifications
Pure Agar	40mm diameter dish
Structured Pure Agar	40mm diameter dish with 4mm diameter indentation
Pure PDMS	40mm diameter dish
Structured Pure PDMS	40mm diameter dish with 4mm diameter indentation
Time	1-24 hr
Oviposition Site Size (Exposure Area)	Through-hole diameter (mm): 0.5, 2, 4, and 8
Oviposition Site Spacing	Interspacing (mm): 0.5, 2, and 4
Agar Sugar Content	5.5g, 11g , and 22g of table sugar
Agar Stiffness	Agar powder (g): 5 (Soft), 10 (Normal), and 20 (Stiff)
Acetic Acid Exposure	Concentration (% v/v): 1, 5, and 15
Zinc Exposure	Concentration (mM): 2, 20, and 70

2.6 Toxicity and Multi-Choice Microfluidic Devices

The toxicity and the multi-choice assay chips were designed and fabricated primarily out of PDMS to allow for controllable delivery of chemicals to oviposition sites. Layers of PDMS were prepared by mixing Sylgard 184 elastomer and curing agent in a 10:1 ratio (Dow Corning Co., Midland, MI, USA), casting over 3D printed master

molds, and subsequently integrating with conventional oviposition agar substrates (see *Section 2.7*) to develop the hybrid agar-PDMS microfluidic devices (Figure 5a). The toxicity assay chip (Figure 5b) contained a single PDMS base channel that was used for dosing a single chemical (zinc or acetic acid) into a designated location (called “oviposition site”) for investigating the technology feasibility and assaying the binary effect of attractive and repelling chemicals on *Drosophila*’s oviposition and viability. The multi-choice assay chip (Figure 5c) contained three PDMS base channels to provide controllable on-chip site dosing with three different chemicals and an objective to assay the oviposition preference (*i.e.* decision-making) of *Drosophila* inside them.

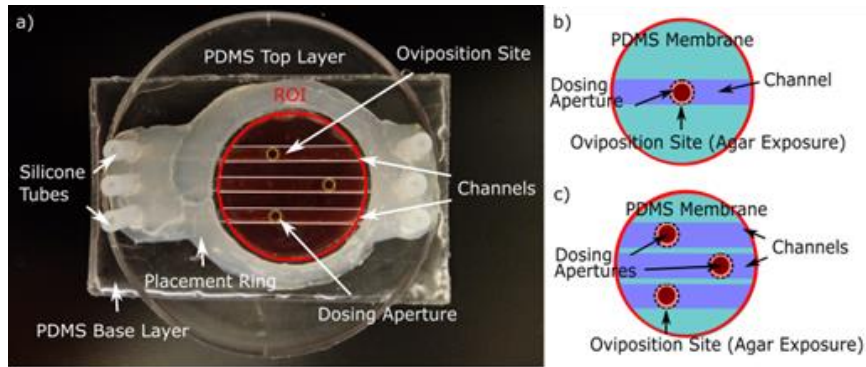


Figure 5: The agar-PDMS hybrid device (a) with the corresponding schematics of the Region of Interest (ROI), as outlined in red, for a clear depiction of the chemical dosing components and the patterned oviposition site arrangements in the toxicity assay (b) and the multi-choice assay (c) chips. Each device consisted of two layers of PDMS fabricated via soft-lithography for creating chemical channels, silicone inlet/outlet tubes, a fly stock bottle placement ring constructed out of hot-melt adhesive, and a patterned PDMS membrane overlaid on agar to define the oviposition sites. As shown, the toxicity chip (b) had one patterned oviposition site positioned on top of the chemical dosing aperture and the channel, whereas the multi-choice assay chip (c) had three.

Both devices (Figures 5b and 5c) consisted of two PDMS layers, an agar layer, a PDMS membrane, and a placement ring for installation of a stock bottle. The PDMS base layer (Figure 5a and 6a) consisted of channels ($400 \times 200 \mu\text{m}^2$ cross-section with a length of 70mm) that were fabricated via soft-lithography using 15ml of PDMS casted on 3D printed molds. The toxicity chip had one channel, and the multi-choice assay chip had three channels that were equally spaced 4.7mm from each other. The top PDMS layer (Figure 6a) included a 40mm diameter dish void in the center, with corresponding dosing aperture(s), for the single- or three-channel devices (Figures 5b and 5c). This was firstly done by spreading 5ml of PDMS pre-polymer over the surface of a petri dish lid (100mm diameter \times 7mm height) with the silicone tubes (2cm-long Masterflex L/S 1/16" tubing) installed in-place. After curing, a dish (40mm in diameter) was laid on top, and 15ml of PDMS was poured into its periphery. This layer was allowed to set, and the lid was removed to expose the formed dish void. One concentric dosing aperture (2mm or 3mm diameter through-hole) or three dosing apertures equilaterally spaced by 17mm from the midpoint were incised with a Harris Uni-Core™ hole-puncher (Ted Pella Inc., Redding, CA, USA) and a 3D printed guide for the toxicity and the multi-choice assay chips, respectively. Overall, this 40mm dish void in the top layer was designed to contain the conventional laboratory agar juice substrate, which was used for the oviposition assays (see next section for integration method).

2.7 Integration of Agar with PDMS via Sacrificial Ice Layer Technique

To integrate agar with patterned PDMS layers in the device, the top and base PDMS layers of the device were first plasma bonded together (Figure 6a). A piece of tape was then used to cover the dosing aperture(s), and water was passed through the base channel(s) (Figure 6b). The device was then put into the freezer (-18° C) until the water in the channel was completely frozen, thereby, creating an impervious sacrificial layer (Figure 6c). The tape covering the aperture(s) was removed after icing, and then 2ml of agar juice solution (detailed in *Section 2.2*) was layered and cured in the 40mm diameter dish void without seeping into the chemical delivery base channel as in Figure 6d. Lastly, the water from the melted ice was syringed out. To confine the oviposition sites and control the amount of chemical exposure through the agar, a 40mm diameter PDMS membrane with patterned 4mm diameter through-holes (proven to be capable of sustaining 45 adult flies and producing pure agar-like oviposition rates [55]) was overlaid on top of the agar substrate (Figure 6e). This PDMS membrane was fabricated via spinning 0.5ml of PDMS pre-polymer on a 6cm×6cm transparency film using a spin coater (MODEL P6700 Series, Speciality Coating Systems Inc., Indianapolis, IN, USA) for 20 seconds at 600rpm. After curing, 4mm through-holes (one single hole for the toxicity assay chip and three holes with 17mm spacing between the centers for the multiple-choice assay chip) were incised via a hole-puncher and a 3D printed guide. These patterns exposed through the PDMS membrane are referred to as the *oviposition sites*.

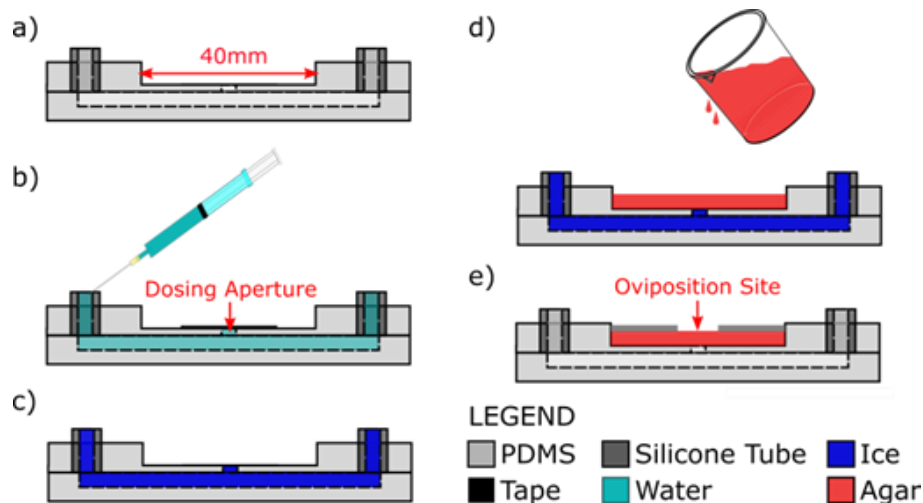


Figure 6: Fabrication methodology to integrate agar into the PDMS-based microfluidic devices. After bonding the device layers (a), water was passed through the channels and frozen as the sacrificial layer (b-c). Agar was poured on top of the device (d). After curing, water was syringed out of the channel(s), and a patterned $d=4\text{mm}$ through-hole (oviposition site) PDMS membrane was placed on top of the agar (e). Using this fabricated device, different chemicals (zinc and acetic acid solutions of different concentrations) were injected into each individual chemical barrel. 45 flies (20 males and 25 females) were subsequently loaded into each stock bottle via the voided channels and left to oviposit for 24hours in the dark. After the assay, the number of eggs inside the oviposition site and over the entire substrate was quantified.

2.8 Experimental Setup in Chemical Dosing and Screening Studies

We conducted two sets of experiments with the devices introduced in previous sections used in the experimental setup shown in Figure 7. Both devices in this paper deliver chemicals to the oviposition sites via hydrostatic pressure of the liquid in the

chemical barrels. The first set of experiments was conducted with methylene blue to characterize chemical delivery properties of our devices. The second set of experiments was performed with zinc and acetic acid on fruit flies to demonstrate the novelty and versatility of this hybrid agar-PDMS device and to determine the effects of these chemicals at different concentrations on *Drosophila* oviposition.

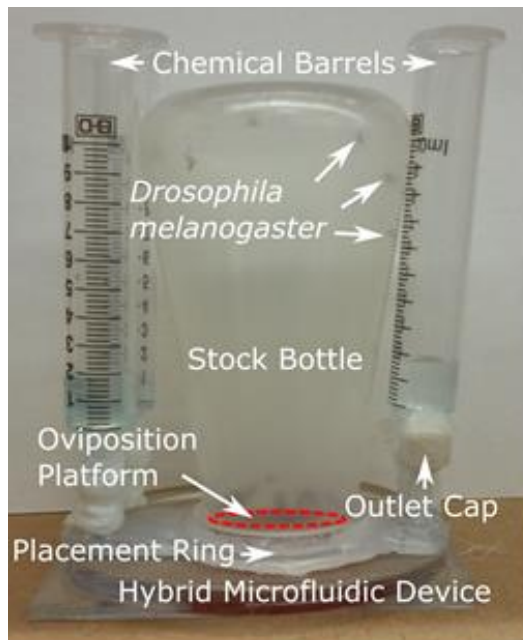


Figure 7: The experimental set-up consisted of syringe barrels for chemical (methylene blue, zinc, and acetic acid at different concentrations) loading into the chip, causing chemicals to infuse up to the oviposition sites via hydrostatic pressure when outlets were capped. Adult flies ($n=45$) were then transferred into the stock bottle, which were capped onto the placement ring, allowing interaction of flies with the oviposition platform (emphasized in red dotted line) and left to oviposit for 24 hours in the dark. The toxicity assay allows for only one chemical dosed via a concentric dosing aperture whereas the multi-

choice assay chip enables three. After the assay, the number of eggs inside the oviposition sites and over the entire substrate was quantified.

2.8.1 On-Chip Chemical Delivery Characterization

To investigate if chemicals would infuse into the agar and to determine the optimal time for the chemicals to reach and fully dose the oviposition site (Figure 5a), a methylene blue solution (1mg/mL) was prepared and used to visualize the rate of chemical delivery through the agar. This experiment was done using the single channel toxicity assay chip (Figure 5b) with chemical dosing aperture diameters of 2mm and 3mm. The column height of the methylene blue in the chemical container (Figure 7), after it was passed through the channel and capped at the outlet tube, was kept at 5cm from the bottom of the device. The experiment was conducted under a microscopy, and the methylene blue spread into the porous agar was imaged every five minutes for the first two hours and hourly thereafter right on top of the oviposition site. The acquired images were quantified by ImageJ to calculate the diameter of the methylene blue plugs delivered.

For the multiple chemical preference assays, to determine if the three chemicals infused into the agar would interact and cross-talk, a similar characterization experiment was done using the multi-choice assay chip with dosing aperture diameters of 3mm (which was proven to provide the optimal swift delivery time from the above characterization). The experiment was conducted as the aforementioned one; however, the set-up was placed under a camera and the spread was imaged for the first two hours in

five minute intervals, followed by hourly till the 10th hour and daily thereon. The spread diameters were then quantified from obtained images using ImageJ. In this experiment, cross-talk (Figure 8a) was defined as the instance at which the methylene blue spread perimeters from the two adjacent dosing apertures touch; and true cross-talk (shown in Figure 8b) was defined as the point at which the spread of one methylene blue reached the boundary of the adjacent oviposition site.

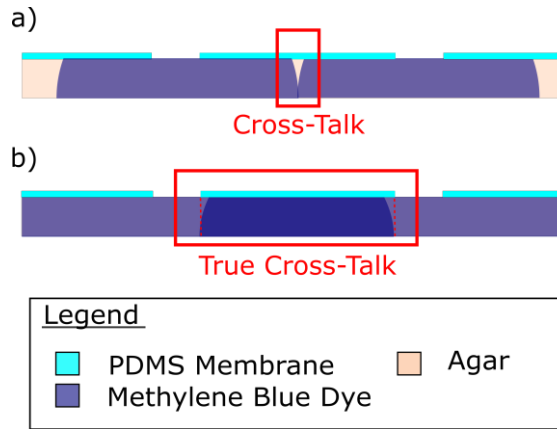


Figure 8: Cross-talk and true cross-talk definition. Cross-talk is defined as the instance, in which the adjacent methylene blue dyes touches each other. True cross-talk occurs beyond cross-talk where the adjacent dye from the dosing aperture reaches the bottom of the oviposition site defined by the 4mm diameter membrane.

2.8.2 Toxicity and Multiple-Choice Preference Assays

The devices were further prepared by attaching chemical barrels (10ml BD syringe) for inputting individual chemicals to the inlets of the fabricated microfluidic devices. After preparing the devices, the toxicity assays were performed via syringing the subjected chemical (*viz.* 2mM, 20mM, and 70mM of zinc solution and 1%, 5%, and 15%

acetic acid solutions) into the chemical barrels, and capping the outlet tube(s). The column height of the chemical from the base of the device was set to 5cm. In these assays, distilled water was used as a control solution in lieu of the prepared chemicals. Firstly, 45 adult flies (25 female and 20 male) were anesthetized and counted into each stock bottle as reported before by us [55]. Then, the stock bottle was placed on top of the 40mm dish void on the device (Figure 7). Subsequently, the devices were placed in an enclosed box and in the dark to allow the flies to oviposit for 24 hours. The start and end times were the same for all the assays to enhance the precision in the oviposition rate by removing the possibility and effects of clock gene dependency [57,58]. At the end of the assay, the viability of adult flies and the number of eggs deposited (*i.e.* oviposition rate) inside and outside of the oviposition sites were quantified via a dissection probe and a microscopy [55]. In the same manner, the multi-choice assays were performed via delivering two concentrations (5% and 15%) of acetic acid and two concentrations (2mM and 70mM) of zinc solution along with control water simultaneously to the chemical barrels of the device to investigate multi-choice decision making. After the assay, the oviposition rate inside the oviposition sites and outside (on the PDMS membrane encircling the sites) were counted; via this, the oviposition site preference index was found by dividing the individual oviposition rates by the total oviposition rate.

Chapter 3

Oviposition Behaviour Investigations of Adult *Drosophila melanogaster* using a Novel Agar Patterning Technique with Polydimethylsiloxane Membranes

D. melanogaster ovipositional assays, studying the effects of physical and chemical substrate factors on egg-laying behaviour, are not highly controlled and lack repeatability and precision due to the use of manual texturing approaches [40,41,44,45,59]. Since they only provide crude results about oviposition behaviour in flies, the PDMS membrane patterning and agar chemical composition manipulation techniques were used to control exposure areas and textures, and to investigate oviposition both in batch format and at a single-egg resolution level. As described in the *Section 2.5*, thin PDMS membranes with patterned through-holes were designed (Table 1) and overlaid on conventional agar-juice plates with various physical and chemical properties (Figure 4), and the oviposition and viability of flies (25 females and 20 males) were quantitatively investigated.

3.1 Survival and Oviposition of *D. melanogaster* on Agar and PDMS Substrates

Surface quality, texture, and chemistry have been shown to have significant effects on flies' oviposition [15,35,36,40,41,43–45,47]; however, the required area of

exposure to agar substrate, in order to keep a population of fruit flies alive and to induce them to lay their eggs naturally (*i.e.* similarly to pure agar substrates) were not investigated. Accordingly, our aim was to quantitatively investigate the effects and the threshold values of surface exposure area on oviposition of fruit flies using our PDMS membrane patterning technique. To achieve this goal, the investigations started by examining the survival and oviposition of fruit flies on pure PDMS substrates in comparison to that on top of the conventional pure agar substrates. For instance, in a representative experiment, from the 45 fruit flies that were left to oviposit on the pure PDMS substrate for 24h, 23 were found dead with only 12 eggs deposited on the substrate (Figure 9a). On the contrary, all the flies survived after 24h on a standard agar plate, and 137 eggs were found to be laid on the substrate (Figure 9b).

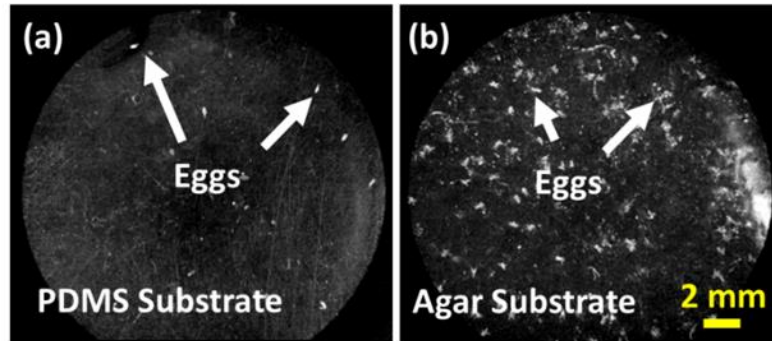


Figure 9: Photographs of oviposited eggs on (a) pure PDMS and (b) pure agar substrates, after oviposition of n=45 flies for 24h. Both substrates showed random oviposition; however, pure agar had a higher ovipositional rate (137 eggs) than the pure PDMS (12 eggs) substrate

A number of T=44 trial experiments demonstrated that on the pure agar juice-plates, many eggs (137.2 ± 15.2 standard error (SE)) could be distributed randomly all over the substrate, whereas eggs were found to be oviposited sparingly (11.9 ± 1.1 SE) on pure PDMS substrates. The corresponding viability rates of the adult flies for the pure agar and pure PDMS substrates were found to be $99.6 \pm 0.2\%$ SE and $50.8 \pm 5.3\%$ SE, respectively. These results clearly showed that the access to agar is vital for full viability of the animals, and that PDMS alone is not an appropriate site for oviposition, or a proper material of choice by itself for fabrication of devices for whole-organism assays. Possible reasons for lack of oviposition on PDMS are the smell of PDMS substrate that was not attractive to *Drosophila*, and its toughness causing obstacles for the ovipositor to be inserted into the media in comparison to pure agar [60]. Lack of water, food, and nutrients is among the reasons for the fatal effect observed on pure PDMS substrates [61,62]. With the understanding of flies' viability and oviposition on pure PDMS and agar substrates, additional experiments could be designed to precisely study the effects of exposure area and size of the oviposition site on these behaviours. In addition, understanding the effect of exposure to PDMS is becoming more and more important in design and development of microfluidic devices for *Drosophila* assays.

3.2 Agar Surface Exposure Area and Its Effects on Survival and Oviposition of *D. melanogaster*

To investigate how much access to agar is required to obtain natural responses (*viz.* survival and ovipositional rates similar to those demonstrated by flies on pure agar substrates), an exposure to agar was introduced and controlled via overlaying of PDMS membranes patterned with various through-holes on top of the standard agar substrate. For instance, when a $d=4\text{mm}$ access hole to agar (corresponding to 1% exposure area) was provided through the PDMS membrane (Figures 10a and 10b), the survival and ovipositional rates of the flies were observed to increase (as compared to pure PDMS) to 100% and 64 eggs, respectively. It was also interestingly observed that the majority of the oviposited eggs (82%) were deposited inside the PDMS through-hole and aligned at the side edges of the hole.

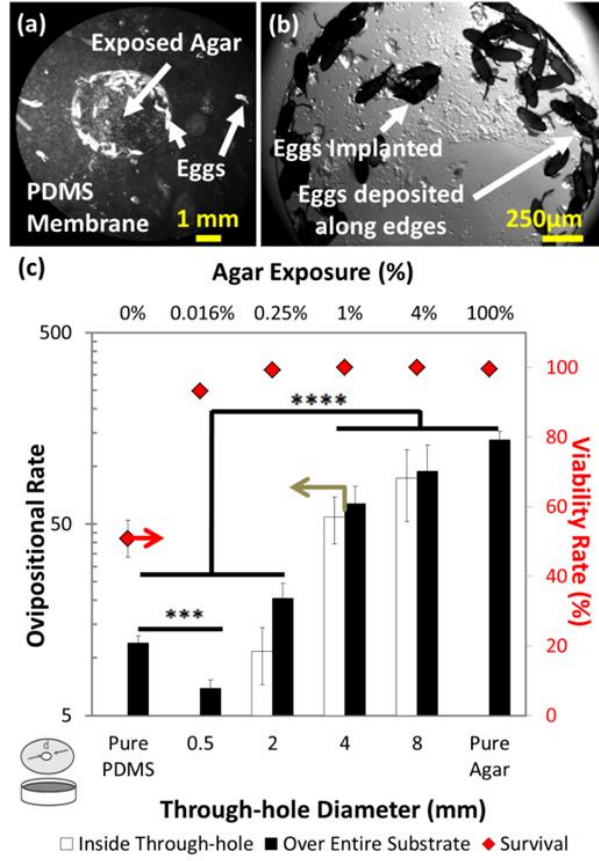


Figure 10: Single through-hole oviposition assay results for 9 trials. (a) A single through-hole substrate ($d=4\text{mm}$) after the oviposition assay. Eggs were found to be deposited inside and along the edges of the through-hole, (b) a magnified image of the oviposition site in (a), (c) the viability and ovipositional rates with standard errors of mean for oviposition inside the through-hole (white column) and on the entire substrate (dark black column). Increasing the exposure area by a $d=0.5\text{mm}$ through-hole (0.016% exposure) increased the survival rate significantly (T-test, $P\text{-value}=3.1\times 10^{-9}$) when compared to pure PDMS substrate. The ovipositional rate over the entire substrate showed a sudden decrease on $d=0.5\text{mm}$ substrates but then increased gradually with an increase in the exposure area. No eggs were deposited inside $d=0.5\text{mm}$ substrates, but the majority (88%) of the eggs were deposited inside the holes on $d=4\text{-}8\text{mm}$ substrates. Agar exposures exceeding 1% ($d=4\text{mm}$) yielded to be statistically significant in ovipositional rate when compared to pure PDMS and $d\leq 2\text{mm}$ substrates (T-test, $P\text{-value}=8.9\times 10^{-13}$).

Figure 10c illustrates the overall survival and ovipositional rate results of T=9 trial experiments for our single-hole oviposition assays with various substrate hole diameters (Table 1). When a 0.5mm diameter (0.016% exposure area) through-hole was introduced, the survival increased from approximately 50% (pure PDMS) to more than 93%, which was significantly different from pure PDMS substrate (P-value of 3.1×10^{-9} using T-test). As the agar exposure area was further increased past 0.016% (to 2mm diameter through-holes and larger), the survival rates reached a plateau showing natural (*i.e.* pure agar-like) survival. Pure agar showed $99.6 \pm 0.2\%$ SE survival in comparison to 1% and 4% exposure areas, which both showed 100% viability. This difference was statistically insignificant and mainly attributed to the occasional death of an animal during our pure agar assays, which may have occurred due to the interruption of excessive egg production and “reproductive overloading” [63]. Statistical T-test analysis performed on agar and substrates with exposure access areas equal and greater than 0.25% ($d=2\text{mm}$) showed no significant survival differences. This behaviour not only validated that agar access is needed for optimal survival, but also showed that only a small exposure of 0.016% was adequate in sustaining most (>90%) of the assayed flies.

In Figure 10c, the oviposition rates over the entire substrate (black columns) and inside the through-hole (white columns) are also presented. The oviposition rate over the entire substrate for 0.016% agar exposure ($d=0.5\text{mm}$ access hole) unexpectedly decreased from our control PDMS substrate, and yielded a significant difference (T-test, P-value= 3.5×10^{-4}) when both were compared. This decrease in the amount of eggs can be

due to possible substrate spatial restriction for oviposition, creating physiological stress on the *Drosophila*. As the agar exposure area was increased further towards 4% ($d=8\text{mm}$), the ovipositional rate on the entire substrate demonstrated an overall increasing trend towards natural (*i.e.* agar-like) oviposition (P-value=0.3 for $d=8\text{mm}$ compared to pure agar substrate). We also noticed a sudden increase in oviposition between the 0.25% ($d=2\text{mm}$) and 1% ($d=4\text{mm}$) exposure areas, which showed a significant difference (T-test, P-value= 8.9×10^{-13}) in the selection of oviposition sites. The 1% exposure area was shown to be adequate for the flies to demonstrate natural oviposition as they would do on a purely agar-based substrate. This can be due to the fact that the adult flies prefer oviposition sites where food and water is prevalent for the survival of the next generation *Drosophila* [29]. Hence, as a natural metabolic response to situations with limited access to food and water (*e.g.* $d=0.5\text{mm}$ holes), flies potentially conserved their energy by decreasing oviposition to increase their starving resistance in correspondence to the coupled-model of reproduction cost-trade off [64,65]. This device may be useful for the future design of sensitive assays where the intake of food, water, and even repulsive chemicals has to be controlled precisely for oviposition investigations.

Comparing the number of eggs oviposited inside (white columns in Figure 10c) the through-holes with the overall number of eggs on the entire substrate (black columns in Figure 10c) provided us with some interesting insights for quantifying and interpreting the site selection behaviour in flies. No eggs were found inside the $d=0.5\text{mm}$ diameter (0.016% agar exposure) through-holes due to spatial restrictions of the site. As the

through-hole diameter was quadrupled; yielding the agar exposure of 0.25%, more than 52% of the total number of eggs were observed to be deposited inside the through-holes, yielding mediocre site-selection behaviour in flies. On the contrary, when the exposures were increased to 1% and beyond ($d > 2\text{mm}$ diameter), $> 85\%$ of the eggs were laid inside the through-holes, showing that site selection was significantly enhanced. In all of these oviposition assays (Figure 10), majority of the eggs that were deposited inside the through-holes were also positioned along the edges of the PDMS membrane and embedded inside the agar base (as seen qualitatively under microscope for the 1% substrate in Figs.10a and b). This trend was true for 72% and 91% of the substrates that had exposure areas greater than 0.016% and 0.25%, respectively. There are a number of advantages associated with our site selection assays that have not been achieved by the conventional oviposition methods. For instance, the 1% and 4% exposure area devices may be used for fundamental investigation of genes that cause defects in oviposition. The 0.016% exposure area device is considered as a sensitive tool that can be used for assessment of other physical and chemical factors that play a role in oviposition as reported in *Section 3.5*.

3.3 Time-Lapse Investigation of Oviposition on Through-hole Substrates

To obtain a better understanding of when oviposition occurs in our assays, and to ensure that flies had enough time to come in contact with the substrate before oviposition,

we performed a series of time lapse studies on pure agar as well as the 1% and 4% exposure substrates at the 12th, 16th, 20th, and the 24th hour. For example, in the pure agar experiment, starting from the 12th to the 20th hour, the ovipositional rate increased gradually with an average of 55.3 ± 10.7 SE eggs. At the end of the time lapse (seen in Figure 11), at the 24th hour, a drastic spike in ovipositional rate was observed (T-test, P-value 4.3×10^{-4}), yielding 248 ± 20 SE eggs.

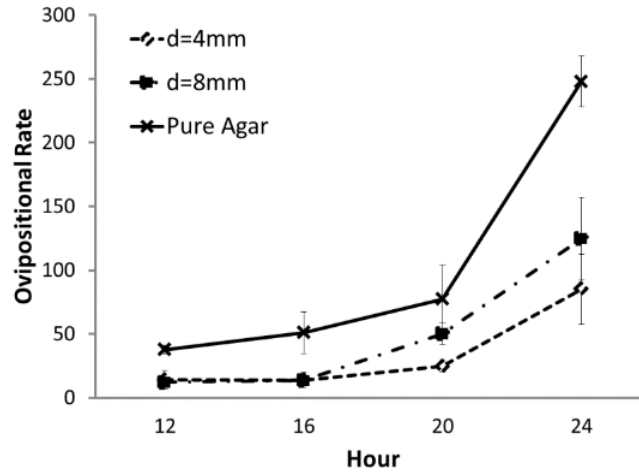


Figure 11: Time lapse investigation of ovipositional rate for the last 12 hours of the assays with pure agar, and single hole ($d=4\text{mm}$ and $d=8\text{mm}$) substrates. In all the substrates, the number of eggs was low and stable until the 20th hour. There was an acute increase between the 20th and the 24th hour in the number of eggs, which was significantly different from the times before.

Similar to pure agar substrates, from the 12th to the 20th hour, the mean ovipositional rates over the 1% ($d=4\text{mm}$) and 4% ($d=8\text{mm}$) exposure substrates remained fairly constant at 17.5 ± 3.4 SE and 25.3 ± 4.8 SE eggs respectively (Figure 11). As the 24th

hour was reached, a significant increase was observed in the number of oviposited eggs (T-tests, P-values= 2.5×10^{-4}). From these observations, the 24 hour assay time period was sufficient enough to allow for our investigations of survival and oviposition of fruit flies in response to surface modifications.

3.4 Effect of Site Size and Spacing versus the Exposure Area on Survival and Oviposition of *D. melanogaster*

Knowing that the physical size of the oviposition site and the exposure area to agar were important factors for full viability of all 45 flies and their natural oviposition behaviour, we sought to investigate for the first time if there were any preferences in oviposition towards these two parameters. Thus, we patterned the agar substrates with 7 holes of $d=0.5\text{mm}$, 2mm , and 4mm diameter (Table 1) and conducted the oviposition and survival assays as discussed before. Each array of through-holes (*i.e.* seven 0.5mm , 2mm , and 4mm diameter) in the substrate corresponded to the exposure areas of 0.112% , 1.75% , and 7% , respectively. This enabled increasing the exposure areas of the substrates significantly as compared to equivalent single-hole assays, while maintaining the physical sizes of the oviposition sites constant. We were also interested in investigating whether site interspacing ($s=0.5\text{mm}$, 2mm , and 4mm) had a significant effect on oviposition. In an experiment, when seven $d=2\text{mm}$ through-holes (1.75% exposure area) with equal inter-spacing of $s=2\text{mm}$ were provided through the PDMS membrane (Figures 12a and 12b), the survival and ovipositional rates of the flies were observed to increase to 100% and 111 eggs, respectively. As shown, the majority of these oviposited eggs (90%)

were deposited inside the PDMS through-holes and along the side edges of the holes. To observe the effects of spacing, the results from the 1.75% exposure substrate ($d=2\text{mm}$; $s=2\text{mm}$) were compared with the same arrayed through-holes but with 0.5mm and 4mm spacing. Similarly, the survival rates for both were 100%, and the ovipositional rates were 132.5 and 122.5 eggs, respectively.

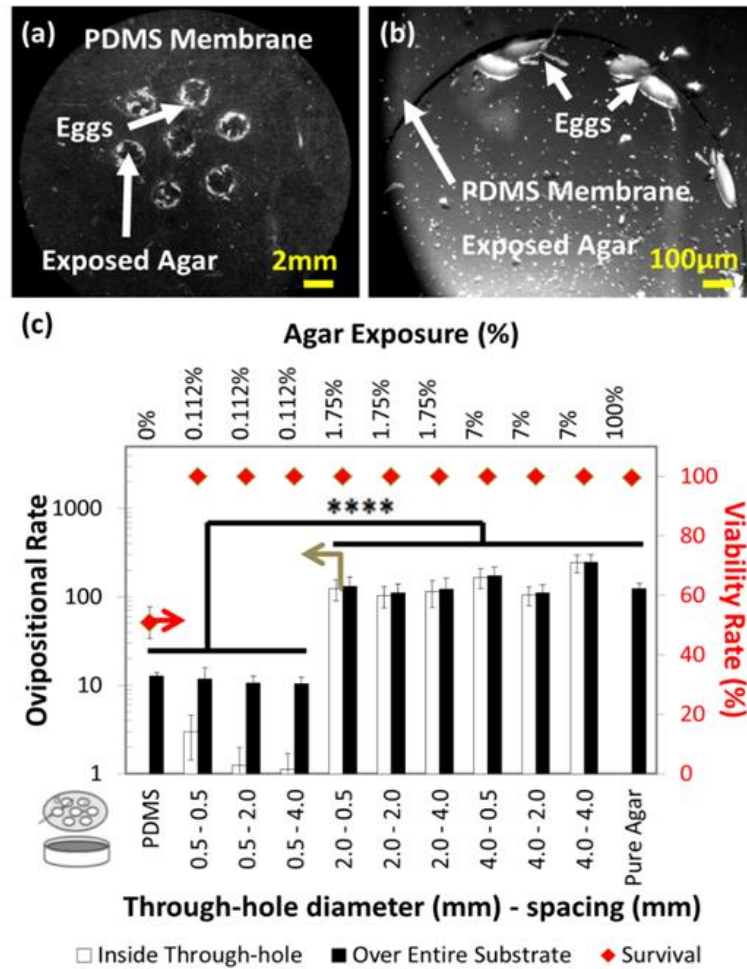


Figure 12: Effect of site spacing on survival and oviposition of *D. melanogaster* (T=7 trials). (a) The spacing assay substrate ($d=2\text{mm}$, $s=2\text{mm}$) under a microscope with one hole magnified in (b). Eggs were laid inside and along the edges of the holes when $d \geq 2\text{mm}$. (c) The overall mean oviposition rate (white columns for eggs laid inside through-hole; black column for eggs on entire substrate) and the viability (diamonds) of flies over various oviposition substrates. Spacing between the sites had no effect on oviposition rate and survival of flies. Agar exposures $>1.75\%$ (seven $d=2\text{mm}$ holes) was statistically significant ($P=7.0 \times 10^{-16}$, T-test) in oviposition as compared to exposure areas of 0.112% (seven $d=0.5\text{mm}$ holes).

Figure 12c shows the overall viability and the ovipositional rate of *Drosophila* on the aforementioned patterned seven-hole substrates (T=7 trials). For all of the assays, the survival rate brusquely increased to 100% as expected. The inter-spacing between the through-holes did not affect the survival rate of animals.

Similar to the agar surface exposure area assay (*i.e.* single-hole experiments in Figure 10), which showed that single $d=0.5\text{mm}$ hole substrates exhibited few to no eggs, the arrayed $d=0.5\text{mm}$ holes (0.112% agar exposure) with spacing of 0.5, 2, or 4mm also showed no significant differences (T-test, $P\text{-value}<0.8$) in ovipositional rates on the entire substrate (dark black columns in Figure 12c). Despite a drastic increase in the exposure area to agar, oviposition rate still did not increase significantly, demonstrating that the physical size of the site played the more important role in oviposition. Furthermore, on the arrayed 2mm through-hole substrates, there was a significant and acute jump (T-test, $P\text{-value}= 5.0\times 10^{-16}$) in ovipositional rate (Figure 12c) due to the increase in energy and the resources available to be spent on reproduction. This substrate showed similarity in oviposition rate to pure agar substrates as opposed to the single-hole 2mm-diameter assays (Figure 10c). This observation demonstrated the importance of exposure area provided that an adequate oviposition site size was available for flies to lay their eggs. Additionally, the varying interspaces showed no effect on the quantity of eggs oviposited over the entire substrates or inside the sites for all the through-hole conditions tested. This suggested that oviposition behaviour is highly dependent on the size of the sites and

the overall exposure area to agar rather than their spatial proximity and the resultant physical constraints to approach the sites.

Figure 12c also shows the effect of exposure area and through-hole inter-spacing on the ovipositional site selection behaviour (white columns compared to the black ones). With a closer examination, $d=0.5\text{mm}$ (0.112% agar exposure) substrates had some random occurrences (47.6%) of very few eggs ($n<3$) being deposited inside the through-holes. Although site selection was still not seen as 95.2% of the eggs were scattered randomly across the substrate, this demonstrated that, by increasing the exposure percentage via six more through-holes, the substrate was deemed slightly more attractive than the single 0.5mm hole. Also, on substrates with seven holes greater than $d=0.5\text{mm}$ (e.g. substrate in Figs. 12a and 12b), most of the eggs (96.7%) were found on the edges inside the arrayed through-holes between the PDMS and the exposed agar interface, leading to a significant increase in site selection in fruit flies (Figure 12c).

3.5 Single-Egg Level Site Selection Assay for Studying the Effect of Agar Properties on Oviposition

Despite providing both texture and exposure to agar, few eggs were laid inside the single (Figure 10c) and arrayed (Figure 12c) oviposition substrates with $d=0.5\text{mm}$ through-holes. Because of this binary observation (*i.e.* egg or no egg) and the convenience in scoring results from this substrate, it was used as the most sensitive tool developed to date to study the effect of other substrate factors on oviposition site

selection at a single-egg resolution level (*i.e.* $n < 5$ eggs per hole). A set of experiments with 1 to 7 through-holes ($d=0.5\text{mm}$) provided confirmatory evidence that increasing the exposure area had no effect on egg deposition inside the 0.5mm diameter holes and site-selection was not achieved (data not shown). To further investigate site selection and to determine whether the oviposition avoidance phenomenon on the aforementioned substrates were due to geometrical constraints of the hole, physical conditions of the substrate base, or chemical composition of the oviposition site, a series of experiments on the sugar concentration and the stiffness of the substrate were conducted as discussed below.

3.5.1 Effect of Substrate Sugar Content on Oviposition Site Selection

To understand the effect of agar chemical composition on oviposition site selection and to investigate if sugar composition is more predominant in oviposition, 5.5g and 22g of sugar were used in preparation of the agar plates as the low and high sugar concentration substrates in reference to 11g in the control assay (used in all other experiments reported in this article). PDMS membranes with seven hexagonally patterned through holes ($d=0.5\text{mm}$, $s=2\text{mm}$) were overlaid on top of these base substrates to produce the sugar modified substrates and the oviposition assays were conducted. The ovipositional rates were similar for all substrates with most of the eggs ($>78.3\%$) deposited randomly outside the through-holes with no preference for site selection (data not shown here). Therefore, altering the sucrose content had no effect on re-stimulating oviposition and site selection.

3.5.2 Effect of Substrate Stiffness on Oviposition Site Selection

Variations in the exposure area and sugar content had no effects on oviposition of flies on $d=0.5\text{mm}$ through-hole substrates. Here, the effect of agar substrate stiffness on the oviposition of flies on the same substrates was studied. As in the previous assays, PDMS through-hole membranes ($d=0.5\text{mm}$) with seven holes were fabricated and overlaid on top of three different agar substrates containing 5g (softer substrate), 10g (standard substrate used in our other assays), and 20g (stiffer substrate) of agar powder to vary their stiffness. After oviposition, the substrates were imaged under a microscope and the mean ovipositional rate inside the through-hole (white columns) and on the whole (dark black column) substrate were quantified for 6 trials (Figure 13).

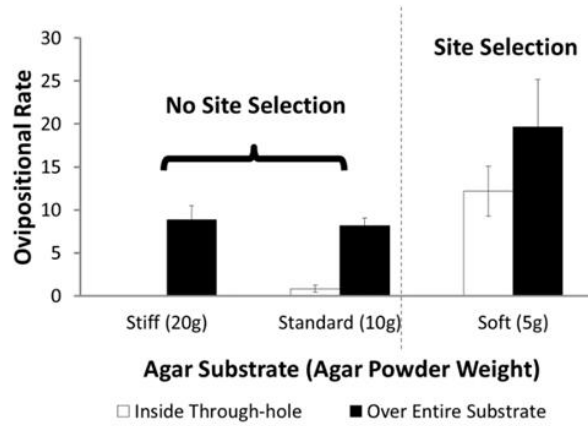


Figure 13: Ovipositional rate inside the through-hole (white column) and on the entire substrate (black column) for seven hexagonally patterned $d=0.5\text{mm}$ holes (with $s=2\text{mm}$) on stiff, standard, and soft agar substrate, which corresponds to the modified agar quantities of 20g, 10g, and 5g ($T=6$ trials). The total mean ovipositional rates on the entire substrate were similar; however, as the stiffness decreased, there was a trending increase in oviposition inside the through-holes. For the soft agar, most of the eggs were deposited inside the through-holes, showing an enhancement in site selection.

As shown in Figure 13, in comparison to the control, the oviposition rates on the entire substrate for the soft and stiff agar platforms were statistically similar (T-test, P-value \geq 0.4). However, when considering the oviposition rate inside the through-holes (white columns in Figure 13), there was an increasing ovipositional trend with the softening of the agar substrate. The stiff substrate exhibited no eggs inside the through-holes while reducing the agar quantity to 10g and 5g yielded 0.8 ± 0.4 SE and 12.3 ± 2.9 SE oviposition rates respectively. Moreover, the corresponding percentages for the number of eggs deposited inside the stiff, regular, and soft substrates were 0%, $10.2\pm5.0\%$, and $61.9\pm24.4\%$, respectively. This could potentially be due the fact that altering the stiffness of the substrate influenced the ability of the flies to insert their eggs deeper into the substrate using their ovipositors. Softer substrates permit more ovipositional activity with lesser effort since *Drosophila* have a tendency to puncture substrates to implant their eggs [66–68]. In addition, all of the through-holes ($d=0.5\text{mm}$ diameter) in the soft substrate had less than 4 eggs per hole due to the small spatial constraints.

These substrates may be used in the future as sensitive tools to study oviposition in details and at a single-egg resolution level. Additionally, they would allow for sorting and collection of eggs in embryonic developmental imaging studies and DNA extraction applications. Moreover, since PDMS is the material of choice in most microfluidic devices, these substrates would provide the flexibility and the possibility to design miniaturized devices for decision-making applications [60] and self-assembly of eggs at

desired locations on a platform for high throughput chemical screening and toxicological studies.

3.6 Effect of Mechanical Structuring on Oviposition Site Selection

We became interested in learning if the oviposition site selection behaviour observed in our agar-PDMS substrates was affected more significantly by the mechanical structure of the hole or by its chemical composition. This investigation was done via patterning a 4mm diameter hole (selected based on the agar-like viability and ovipositional rates as reported in Figure 10c) in pure PDMS (called “*conditioned PDMS*”) and pure agar (called “*conditioned agar*”) substrates and the conductance of standard ovipositional assays as discussed in the *Section 2.5*.

As seen in Figure 14, both homogenous substrates, whether conditioned or unconditioned, exhibited similar viability rates, demonstrating that mechanical structuring did not contribute to altering the viability of 45 adult flies in the assays. The oviposition rates for the unconditioned pure PDMS and the pure agar substrates were 9.4 ± 1.5 SE and 232.3 ± 18.4 SE respectively. Oviposition on pure PDMS was found to be statistically similar to the *conditioned PDMS* substrate (T-Test, P-value=0.4), and no oviposition inside the PDMS holes was observed. The *conditioned agar* demonstrated some minor significance in overall oviposition compared to the pure agar (T-Test, P-value= 4.0×10^{-2}). This demonstrated that the agar exposure along with surface texture made the substrates slightly more desirable. However, looking at the ovipositional rate

inside the through-holes, *conditioned agar* only had 19.7 ± 17.0 SE eggs (~6% of the overall oviposited eggs) laid inside the hole. This showed that mechanical structuring alone was not sufficient to induce site selection in fruit flies. Overall, our experiments show that in order for site selection to occur, the substrate surface ought to have a component that repels the flies (*i.e.* the patterned PDMS membrane), a component that attracts the flies (*i.e.* agar-juice base surface), and a mechanical texture (*i.e.* PDMS-agar step interface).

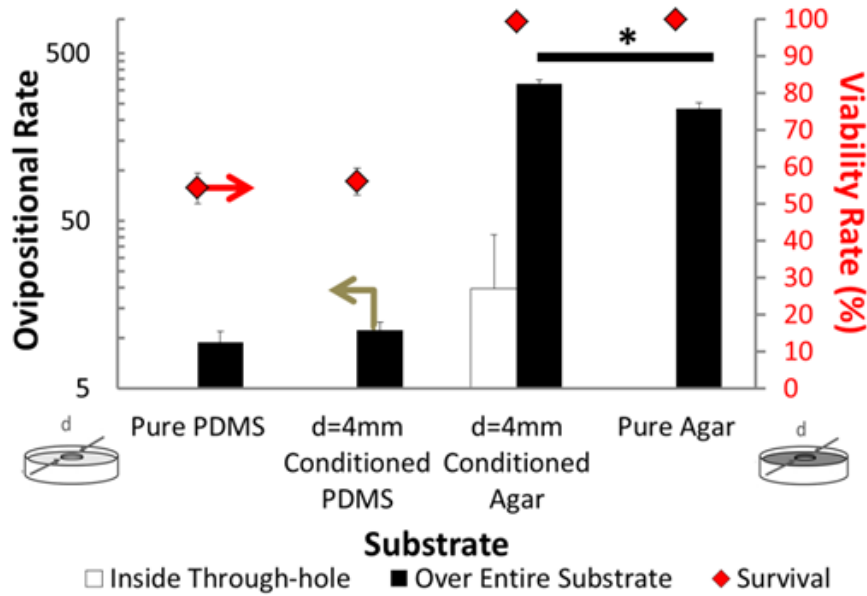


Figure 14: Viability and ovipositional rate inside the through-hole (white column) and on top of the entire substrate (black column) for pure PDMS, $d=4\text{mm}$ conditioned PDMS, $d=4\text{mm}$ conditioned agar, and pure agar substrates ($T=10$ trials). Both pure and conditioned substrates had similar survival rates. The number of eggs deposited in total was similar in PDMS substrates; however, conditioned agar showed more eggs than pure agar yielding a difference ($P\text{-value}=4.0 \times 10^{-2}$). Despite patterning the substrate in the conditioned agar assays, site selection was not observed for flies.

Chapter 4

Integrated Hybrid Microfluidic Devices for Oviposition-Based Dosing and Chemical Screening in Adult *Drosophila melanogaster*

Present whole-organism assays studying the effects of chemicals on *Drosophila* oviposition [20,35,36,55,60,69,70] are time-consuming and labor-intensive due to off-chip dosing preparation and lack localization for egg quantification after the assay. Moreover, current methodologies introduce more errors via the creation of extra textures, which causes more oviposition to occur, and are inflexible in providing controllable and easy chemical screening with the possibility of integrating with current micro-technologies. Therefore, using the fundamental oviposition studies done in the aforementioned sections with the devised agar-PDMS technique, we have designed and successfully tested two types of controllable chemical dosing microfluidic devices, *i.e.* toxicity assay chip and multi-choice assay chip, which allowed for chemical screening of free-flight *Drosophila*'s oviposition and decision-making between different chemicals. The infusion rate of the chemicals (*e.g.* water, zinc solution, or acetic acid solution) in the device was experimentally characterized by using a methylene blue dyed solution (to enhance visualization) under two chemical aperture sizes (*i.e.* $d=2\text{mm}$, 3mm) in the

single-channel toxicity assay device. Using the optimal aperture size found, the toxicity and the multiple-choice assays were conducted as described in *Sections 2.6 and 2.8*.

4.1 On-Chip Chemical Delivery Characterization with Methylene Blue

To investigate if chemicals could be hydrostatically infused into agar via the fluidic channels and to determine the optimal dosing aperture size and the time window required for chemicals to reach the top of the agar layer and fill the entire $d=4\text{mm}$ exposure area (*i.e.* oviposition site), methylene blue was used as a representative chemical. Accordingly, toxicity assay chips (single-channel) with 2mm and 3mm diameter dosing apertures were fabricated and tested. As described in *Section 2.8.1*, a 1mg/ml methylene blue solution was injected into the device to achieve a 5cm-height column in the chemical barrel. The solution was allowed to controllably infuse into the device for up to 24h. The spread of the dye at the dosing aperture was imaged in 5 minute intervals for 2 hours and hourly thereafter. For demonstration purpose, Figure 15 shows the methylene blue spreads in a multi-choice assay device that was similar to the single-channel device.

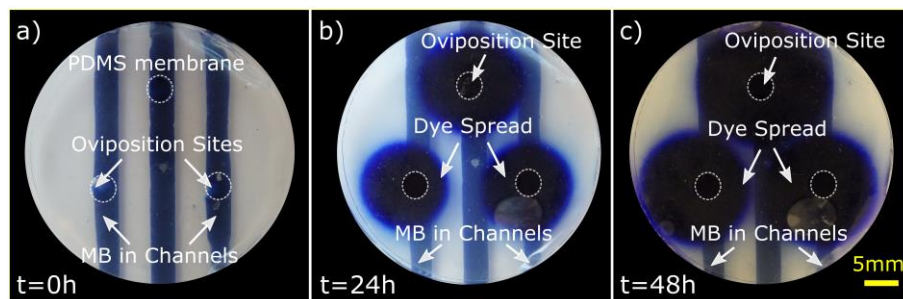


Figure 15: Time lapse images of the methylene blue (MB) infusion experiment at 0 h (a), 24th h (b), and 48th h. At 24th h (the timing used in oviposition assays) the spread of methylene blue were conserved and the edges did not touch (no definite cross-talk of chemicals). At 48th h, the edges of the spread touched (indicating chemical cross-talk). However, since the dye spread was less than 17mm in diameter, true cross-talk did not happen, which demonstrated that the chemical composition of the oviposition sites was unaffected.

The images attained were quantified with ImageJ software (as described in *Section 2.8.1*), firstly, by edge detection, followed by the measurement of the area via the circle selection. Converting the measured areas into diameters, the infused dye profiles were obtained for three trial experiments (Figure 15a). Since the thickness of the agar in the device was ~1mm, the spread diameter ought to be at least 6mm to ensure that the dye had infused to the surface of the agar and covered the entire 4mm-diameter oviposition site. The methylene blue dye spread in the device with the 2mm aperture reached a diameter of 6mm within ~3.6h and slowly expanded to ~9mm in 10h. In comparison to the 2mm opening, the rate of the dye spread in the 3mm dosing aperture device was decreased significantly by half, taking only 1.8 hours to reach 6mm. This spread

expansion beyond the 6mm mark had no effect on the assay since the chemical exposure to the flies beyond the 6mm diameter was prevented by the patterned PDMS membrane on top of the agar.

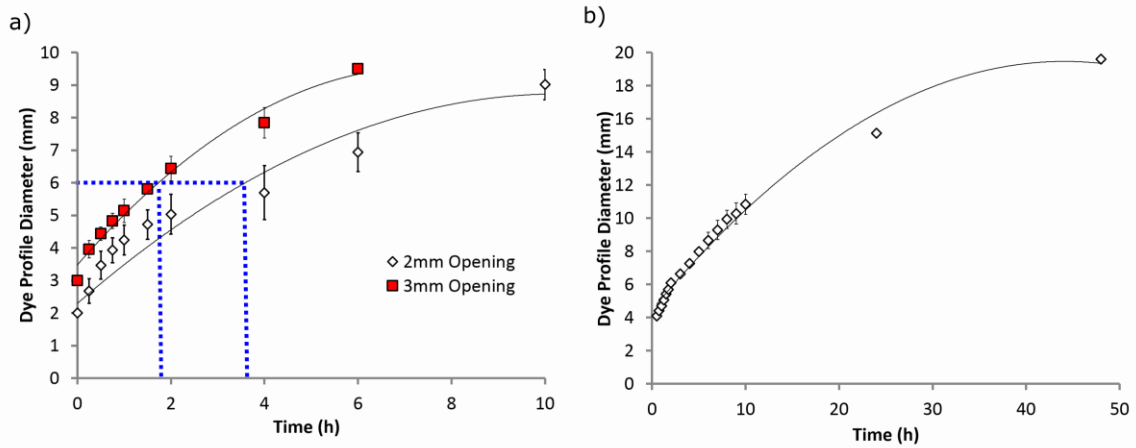


Figure 16: Time lapse investigation of methylene blue spread (a) using dosing aperture sizes of 2mm (white diamonds) and 3mm (red squares) in the toxicity assay chip, and the cross-talk time lapse investigation (b) using the multi-choice assay chip with $d=3\text{mm}$ dosing aperture for 3 trials with its standard error of mean. The overall polynomial trend lines of second order were fitted onto both graphs. As shown with the blue dotted-lines in (a), 3mm aperture provided approximately 2 hours for solution to reach the top surface of the oviposition site which was sufficient for fly transfer into the device and fly recovery from anaesthetisation. On the other hand, in (b), given three 3mm apertures and 24 hour assay periods, the solution spread diameter remained under 16mm, which was less than 17mm and 34mm (diameters required for cross-talk and true cross-talk). Cross talk happened at $\sim 26^{\text{th}}$ h whereas true cross-talk, defined as the spread that reaches the bottom of the 4mm oviposition site, did not occur at all.

In our chemical screening assays, the 45 adult flies were anesthetized and transferred into the microfluidic devices. Prior to the assay, since the flies were also given an hour to recover from the anesthesia [55], the optimal desired rate of chemical delivery to cover the entire oviposition site (that may be seen via the 6mm spread at the opening) plus the experimental preparation time ought to be around two hours. According to our results (Figure 16a), the time it took the 2mm aperture was 3.7h while increasing the dosing aperture size to 3mm was observed to yield a better time range for the reagent preparation and dosing of the oviposition site prior to the recovery of the adult flies after anesthetization. This time window provided by $d=3\text{mm}$ aperture was the optimal time that allowed for the loading of the chemical into the device within an hour before transferring in the animals. In essence, 3mm was the most efficient time range for the reagent preparation, and the dosing of the oviposition site prior to the recovery of the adult flies after anesthetization; thus, the toxicity assay and the multi-choice assay devices were fabricated with 3mm apertures.

Using the multi-choice assay chip, a possible issue foreseen was the cross-talking of the chemicals (Figure 8 and Figure 15c). Therefore, a cross-talk characterization test by methylene blue was performed to verify that chemicals indeed do not interact and mix within the given 24h assay period. As in Figure 5c, the 3mm dosing apertures were situated at an equidistance of 17mm to provide maximum distance and minimize cross-talk. Three trial experiments were conducted and the methylene blue spreads were imaged and quantified similarly. Experimental results were averaged and exhibited in

Figure 16b. From the trend line fitted, cross talk (as defined in *Section 2.8.1* and Figure 8 as the instant when the dyes touch; that's to say that the average of the three diameter dye spread is at 17.32mm) happened at ~26th hour. This means that during the time of our 24 hour assay period, none of the chemicals will meet each other (Figure 16b). On the other hand, true cross talk (where the dye from one aperture reaches the boundary of the 4mm oviposition site) would never happen since dye profile almost plateaus at $d=20\text{mm}$ even after 40h. Therefore, it was verified that chemicals infused through the agar will not mix within a 24 hour window, and prolonged assay using this device will not change the chemical content of the oviposition site.

4.2 Toxicity Assay Chip and Concentration-Dependent Oviposition in Fruit Flies

Using the single channel microfluidic device (Figure 5b) with a $d=3\text{mm}$ dosing aperture, zinc and acetic acid at various concentrations [35,36] were individually assayed to investigate the influence of each reagent on adult *Drosophila* viability and oviposition. This assay sought to determine if adult flies are capable of sensing the concentration of chemicals in the novel miniaturized chemical screening device when selecting sites for oviposition. Moreover, this assay was aimed to examine if this novel chip was able to correlate the amount of oviposition with the degree of chemical exposure and to define the threshold concentration ranges where the oviposition response changes from attraction to avoidance.

4.2.1 Effects of Zinc on Fly Viability and Oviposition

Zinc solutions at 2mM, 20mM, and 70mM concentrations were inserted into the chemical barrel of the devices (Figure 7) to dose the oviposition sites within 2 hours before the start of adult flies' recovery from anesthesia. Distilled water was used as the control solution in this assay. The flies were left to oviposit for 24 hours in the dark and in an enclosed box as previously reported [55]. Since the oviposition rate is also dependent on clock genes [57,58], all the trials were purposely done in the dark and also started and ended exactly at the same time of the day. The survival of adult flies and the amount of eggs oviposited inside the 4mm through-hole oviposition site and over the entire substrate of the device were quantified with the aid of microscopy as described in *Section 2.8.1*. For instance, after the 24h assay period with 2mM zinc, the adult flies' viability was 100%, and the amount of eggs deposited inside the oviposition site (Figure 17a and 17b) and over the entire device were 45 and 50 eggs, respectively.

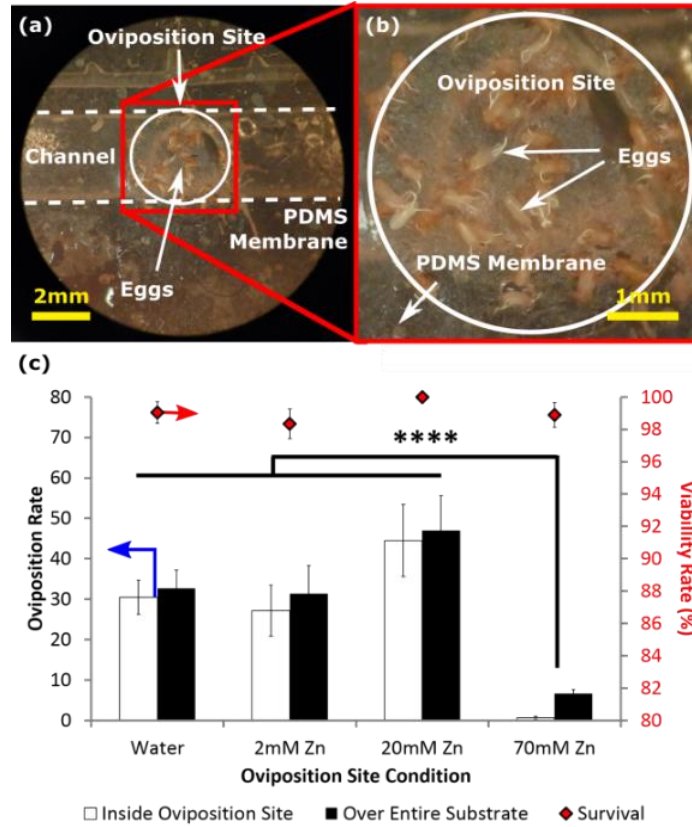


Figure 17: Toxicity assay results for zinc (2, 20, and 70mM) with (a) microscopy image of the 4mm oviposition site after the 24h assay with 2mM of zinc exposure via a single channel (shown in the white dotted lines). Eggs were found to be deposited mostly inside the oviposition site as magnified in (b). The oviposition rate, shown in (c), inside the $d=4\text{mm}$ oviposition site (white bars) and over the entire substrate (black bars) of the hybrid device with standard errors of mean for 6 trials ($T=6$) are also shown. Most eggs were oviposited inside the sites with water and 2-20mM zinc whereas oviposition on 70mM zinc was significantly decreased. 70mM oviposition occurred mostly outside of the through-hole (thereby, showing no site selection). The oviposition rate inside the through-hole for 70mM of zinc was significantly different from the 0 and 2mM with respective P-values of 4.0×10^{-5} and 7×10^{-4} via T-test. The viability (red diamond, 2nd Y-axis) shows the percentage of adult flies that survived after the 24h assay. All conditions were statistically similar with viability rates greater than 93%.

Figure 17c shows the overall viability and oviposition rate results for T=6 trial experiments with zinc at various concentrations. As exhibited, the viability rates (shown in red diamonds) for all the concentrations of zinc were found to be statistically similar and greater than 93%. This showed that the exposure to zinc via the oviposition site for a 24 hour period had no negative effect on the adult flies' viability. It also demonstrated that the toxicity assay device was capable of sustaining adult flies and can be used with any compatible chemicals for viability assays on the chip.

The oviposition rate inside the 4mm diameter oviposition site (white columns in Figure 17c) is also exhibited for each molar concentration of zinc. For the 2mM and 20mM zinc assays, the oviposition rates inside the sites were found to be statistically similar to the control assay with distilled water. In contrast, 70mM of zinc was observed to have an average oviposition rate of 0.7 ± 0.4 SEM, which was statistically different from the control, 2mM and 20mM zinc assays (T-test, $P\text{-value} = 2.6 \times 10^{-7}$). This showed that the concentration of zinc plays an important role in inducing attractive or deterrent behaviours in flies. Moreover, by comparing the number of eggs oviposited inside the sites (white columns in Figure 17c) to the quantity of eggs deposited over the entire substrate (black columns in Figure 17c), a quantitative data called *site selection* [55] in this paper was also investigated. Most of the eggs (>85%) were found to be deposited inside the dosed oviposition sites of the control, 2mM and 20mM of zinc assays, whereas less than 11% of the eggs were found to be deposited inside the oviposition sites dosed with 70mM of zinc, yielding no preference in the flies to select these sites for egg laying

(*i.e.* no site selection). In our assay, both site-selection and the amount of deposited eggs in the oviposition sites dosed with 20mM of zinc were found to be slightly higher than the 0 and 2mM sites. This demonstrated that 20mM of zinc may potentially possess a higher degree of attraction relative to the other two reagent concentrations, which can be investigated upon design and development of more sensitive devices (with smaller oviposition sites for single-egg assays) as reported earlier by us [55]. Because of such capabilities, our novel microfluidic device can be used for the investigation of zinc concentration threshold ranges where behavioural changes from attraction to repulsion can be detected quantitatively. This ought to be in between 20mM and 70mM as resulted from our preliminary experiments (Figure 17c).

4.2.2 Effects of Acetic Acid on Fly Viability and Oviposition

The effect of acetic acid at 1%, 5% and 15% concentrations was also assayed on flies within 24 hours of exposure as discussed in *Section 2.8.1*. The oviposition rate inside (Figure 18a and 18b for 5% acetic acid) and over the entire substrate were quantified along with the adult flies' viability for T=6 trial experiments.

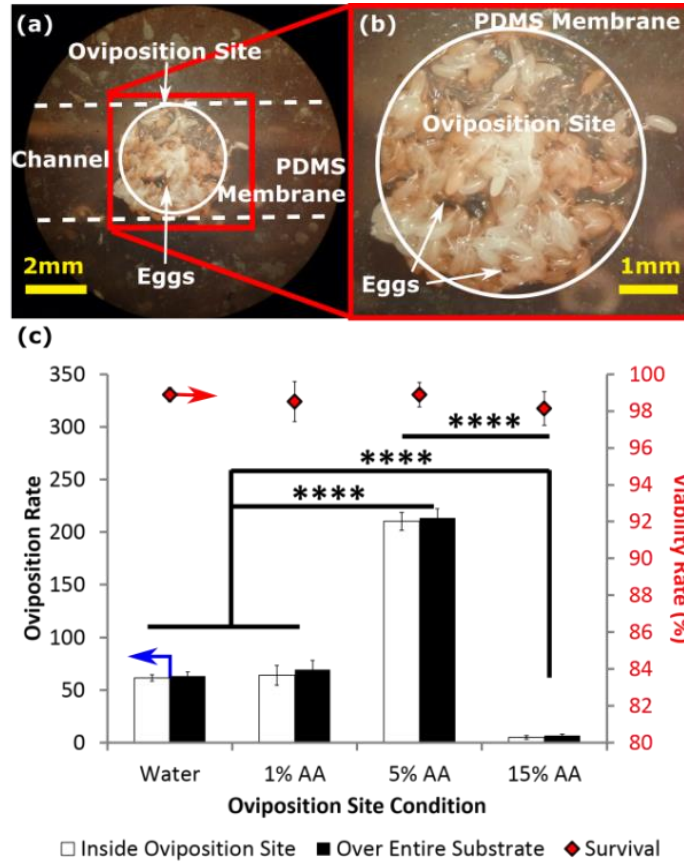


Figure 18: Toxicity assay results for acetic acid (1%, 5%, and 15%) with (a) microscopy image of the 4mm oviposition site after the 24h assay with 5% of acetic acid exposure via a single channel (white dotted lines). Eggs were found to be deposited primarily inside the oviposition site, as magnified in (b). Oviposition rate inside the $d=4\text{mm}$ site (white bars) and over the entire substrate (black bars) of the hybrid device with standard errors of mean for 6 trials ($T=6$) of acetic acid are shown in (c). 5% acetic acid exposure showed great attraction in oviposition, whereas 15% exposure showed strong avoidance. Acetic acid between 0 to 1% was found to be significantly different from 5% and 15% (P -values of 7.0×10^{-5} and 1.5×10^{-5} , respectively). As seen, the viabilities of flies at all concentrations were similar and greater than 93%.

The adult flies' viability (red diamonds in Figure 18c) for all acetic acid concentrations were found to be statistically similar yielding approximately 100%. This demonstrated that the acetic acid exposures within 24 hours had no negative impact on the adult flies. Together with the viability results of the zinc assay, it can be concluded that the toxicity assay chip is capable of sustaining adult flies and useable with any compatible chemicals for viability-based toxicity assays on a chip. In this manner, this device can also indicate if a chemical is extremely toxic or lethal to the flies for one day via the quantification of adult fly survival rates.

The oviposition rate inside the 4mm diameter sites for 1%, 5%, and 15% acetic acid concentrations in comparison to pure water (the control) is shown in Figure 18c. In 1% acetic acid exposure, the oviposition rate was found to be statistically similar to the control. We observed a significantly higher oviposition rate (T-test, $P\text{-value}=3.1\times 10^{-7}$ compared to water and 1% acetic acid) when 5% of acetic acid was provided to the flies in the oviposition site. In contrast, when acetic acid concentration was increased further to 15%, oviposition on the substrate was observed to have an exceedingly low average of 5 ± 1.7 eggs. This was statistically different from the control and 1% assays (T-test, $P\text{-value}=2.4\times 10^{-8}$), as well as the 5% assay (T-test, $P\text{-value}=1.2\times 10^{-6}$). This reconfirmed that oviposition was highly preferred at the acetic acid concentration of 5% as in corroboration with the experimental study done by Joseph et al [35]. Our studies in a miniaturized chemical screening device also showed that concentration of acetic acid significantly influences the oviposition behavior of the flies, shifting the response of the

animals from strong attraction to complete avoidance of the site when the concentration is increased from 5% to 15%.

In addition, to investigate if oviposition site selection occurred, comparisons were made between the number of eggs inside the sites (white columns in Figure 18c) and over the entire substrate (black columns). Most of the eggs (>91%) were oviposited inside the dosed sites of the control, 1%, and 5% acetic acid assays, demonstrating the site selection phenomenon. However, only 5.0 ± 1.7 SEM eggs were found inside the sites of the 15% acetic acid assays, which accounted for less than 60% of the total amount of eggs oviposited on the entire substrate, and hence a moderate degree of site selection. In comparison to the 70mM repellent concentration of zinc in Figure 6c, although oviposition was avoided in both of the environments, a relatively higher degree of site-selection was observed on 15% acetic acid substrates. Flies were observed to override their positional repulsion to acetic acid during the need to oviposit [35]. We deduce that this is true at lower concentrations of 1% and 5%. At higher concentrations, acetic acid overriding does not occur. This is possibly due to the over-whelming and stimulated olfactory senses [35].

4.3 Multiple-Choice Assay on a Chip

From the above toxicity assays, it was found that oviposition attraction and deterrence is chemical concentration-dependent, and given a single attractant, most of the eggs were deposited inside oviposition sites as opposed to the peripheral PDMS

membranes, creating a distinct binary observation in our device. Because of these observations, we sought to further investigate if similar behaviours would occur under simultaneous exposure to multiple concentrations of a chemical. Thereby, we developed a new assay with two additional chemical inputs using the multiple-choice assay chip. This provided a more complex environment and necessitated a more sophisticated decision-making process in the flies which was studied via a systematic series of experiments.

4.3.1 Oviposition Preference in Different Zinc Concentrations

Distilled water (the control), 2mM, and 70mM zinc solutions were injected into the syringe barrels to reach a column height of 5cm from the bottom of the multi-choice assay chip, and the oviposition assay was conducted as detailed in *Section 2.8.2*. Subsequently, the viability of adult flies and the amount of eggs inside the 4mm sites (Figure 5c) and on the surrounding PDMS membrane of the device were quantified. For example, in a representative zinc experiment (shown in Figure 19a) after the assay period, the adult flies' viability was 100%. Moreover, the amount of eggs deposited inside the control (Figure 19b), the 2mM zinc (Figure 19c) and the 70mM zinc (Figure 19d) oviposition sites were 28, 76 and 5 eggs, respectively. On the PDMS membrane, 4 eggs were oviposited sparsely at random locations. This corresponded to the oviposition site preference indices of 0.3, 0.7, and 4.0×10^{-2} for the control, 2mM, and 70mM of zinc, respectively.

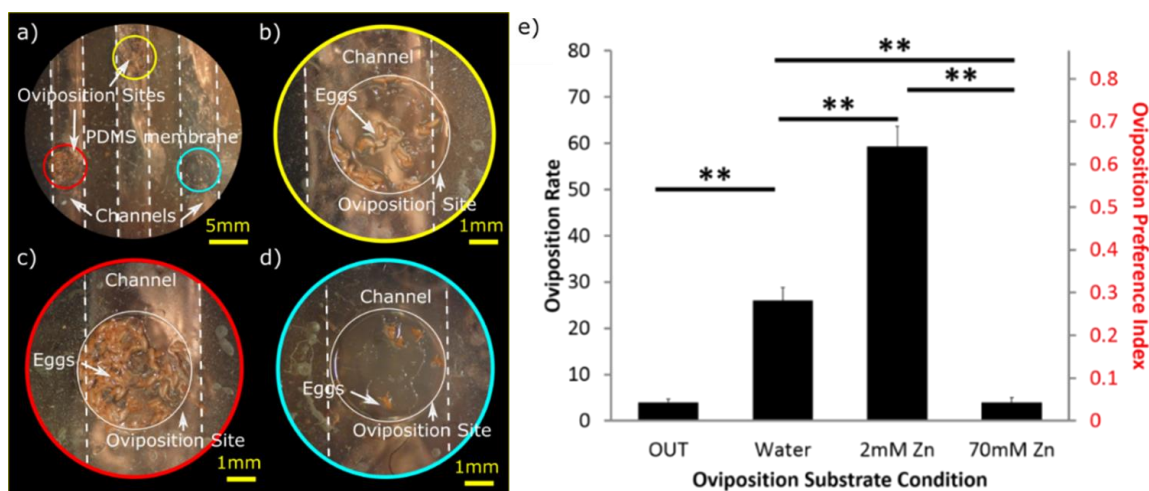


Figure 19: Multi-choice assay results with zinc (Zn) and the microscopy images of 4mm oviposition sites outlined in yellow, red, and blue for control (b), 2mM Zn (c), and 70mM Zn (d) after the 24h exposures via chemical channels (shown by white dashed lines). From the magnified pictures, eggs were found to be deposited mostly inside the oviposition sites. Average oviposition rates and preference indices inside and outside of the sites with standard errors of mean for 3 trials is shown in (e). As opposed to the single-channel results where no preference was identified between water and 2mM Zn, flies showed a higher preference towards 2mM of zinc (T-test, $P\text{-value}=4.1\times10^{-3}$) that was statistically different from sites dosed with water or 70mM Zn (T-test, $P\text{-value}=7.96\times10^{-3}$). The 70mM exposure still exhibited strong avoidance.

Figure 19c shows the oviposition rates and site-selection preference indices for T=3 trial experiments (with 100% viability) of the multi-choice assay with zinc. In the 4mm diameter oviposition sites infused simultaneously with 2mM and 70mM of zinc, the average oviposition rates were found to be approximately 59 and 4 eggs, respectively. The corresponding oviposition preference indices were 0.6 and 4.0×10^{-2} . These were significantly different in comparison to the water control experiment, which yielded 26

eggs and 0.3 in oviposition preference index. As opposed to the single-site toxicity assay results (Figure 17) where no oviposition preference was identified between water and 2mM zinc exposures, the 2mM of zinc was found to be the most preferred site with the highest oviposition rate in the multi-choice assay device, yielding a statistical significance when compared to water (T-test, $P\text{-value}=4.1\times 10^{-3}$). Therefore, different oviposition behavioural responses could be detected in our devices when chemical concentrations were assayed independently or simultaneously on fruit flies. The 70mM zinc sites were still strongly avoided by the flies (*i.e.* no site selection) with a statistical difference yielding a $P\text{-value}$ of 7.9×10^{-3} via T-test. Likewise, the PDMS membrane surrounding the oviposition sites yielded 4 eggs and 4.0×10^{-2} in preference index, showing deterrence and a similarity to 70mM of zinc. This site repellence and random dispersion of eggs was in agreement with our previous study on controlled agar exposure assays [55] and the aforementioned single-site zinc assays conducted in the toxicity device.

4.3.2 Oviposition Preference in Different Acetic Acid Concentrations

Distilled water (0%), and 5% and 15% acetic acid (AA) solutions were simultaneously tested in our multi-choice assay chip. The viability of the flies in all 4 trials was 100%. For instance, in an experiment, the amount of eggs deposited inside the control (Figure 20b), 5% AA (Figure 20c), and 15% AA (Figure 20d) oviposition sites was 30, 41 and 0 eggs, respectively. Five eggs were found to be sparsely deposited outside of the oviposition sites at different locations on the PDMS membrane. This

corresponded to the oviposition site preference indices of 7.0×10^{-2} , whereas the control (water), 5%, and 10% acetic acid exhibited 0.4, 0.5, and 0, respectively.

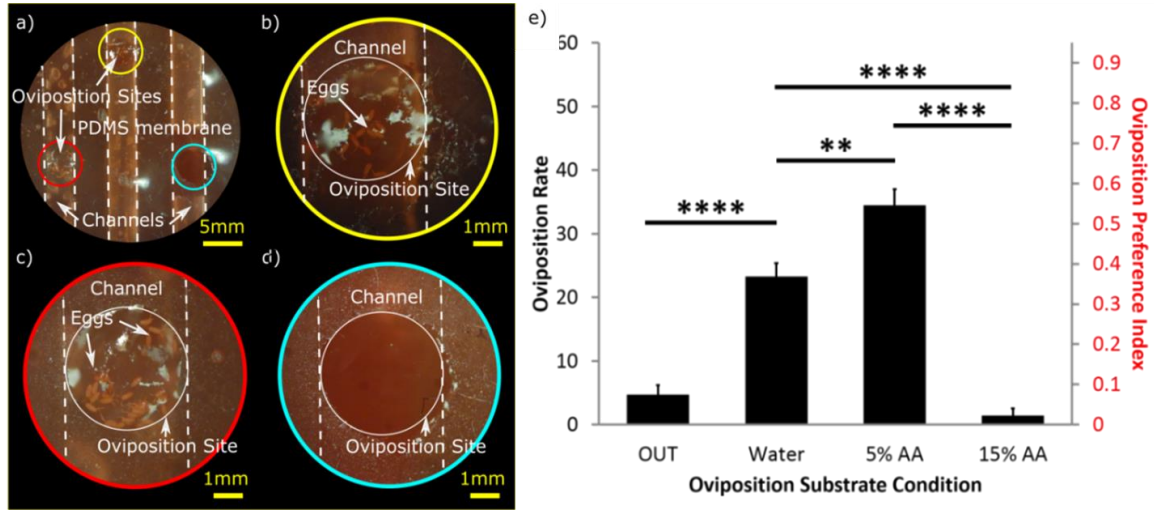


Figure 20: Multi-choice assay chip results with 5% and 15% acetic acid (AA). Microscope images show all three oviposition sites (a) outlined in yellow (water (b)), red (5% AA (c)), and blue (15% AA (d)) after the 24 h assay. White dashed lines indicate chemical delivery channels. Average oviposition rates (black bars corresponding to the primary black axis) and preference indices inside and outside of the sites (black bars corresponding to the secondary red axis) with standard errors of mean for 4 trials is shown in (e). The 5% acetic acid sites were found to be more preferable whereas 15% was significantly deterrent with respect to the control (with respective P-values of 2.4×10^{-3} and 4.0×10^{-5} via T-test).

Figure 20e shows the average oviposition rates and site-selection preference indices for the four acetic acid multi-choice assay trials. The average number of eggs oviposited in the 4mm diameter sites doped with 5% and 15% acetic acid were 34.5 ± 4.7 eggs and 1.4 ± 0.9 eggs, yielding the corresponding oviposition preference indices of 0.5 and 2.0×10^{-2} respectively. Moreover, the control and the surface of the PDMS membrane

contained 23.3 ± 2.5 SE eggs and 4.9 ± 1.3 SE eggs respectively, which corresponded to oviposition preference indices of 0.4 and 7.0×10^{-2} . It was found that, in comparison to the control, 5% acetic acid had the highest amount of oviposition and was the most attractive site, whereas 15% acetic acid was the least favorable and therefore avoided (with no site-selection); both results were significantly different from each other and the control, exuding P-values of 2.4×10^{-3} and 4.0×10^{-5} for 5% and 15% acetic acid via T-test. These results obtained in a miniaturized chemical screening device corroborate well with the studies done by Joseph et al. who used a conventional oviposition assay platform [35].

In comparison of the total amount of eggs oviposited on our substrates and that of our previous studies [55] which all yielded >120 eggs, the lesser number of eggs in this study can be attributed to the longer assessment period of oviposition sites and learning [60,71] since 3 different concentrations of chemicals had to be explored by the flies. Thus, in conjunction with all the above assays, the hybrid agar-PDMS device fabricated for chemical dosing and screening have proven to be an excellent platform to assay adult *Drosophila*'s response to different chemical concentrations and the biological learning in a microfluidic environment.

Chapter 5

Conclusion and Future Direction

5.1 Summary of Thesis

Current physical and chemical substrate studies lack accuracy and precision in control and repeatability due to the current methodology for oviposition substrate preparation. The conventional way of preparing oviposition petri dishes is labour-intensive, manual, time-consuming, inflexible for on-chip dosing of chemicals, and subjected to chemical concentration inconsistencies due to evaporation and the lack of a chemical supply source. Moreover, these substrates are large in size with low number of chemically-dosed oviposition sites, hence not useful for advancement into high throughput assay devices.

In the first part of this thesis, a novel miniaturization-based technique for investigating oviposition was developed. Using this novel technique we have successfully conducted a series of parametric assays (at collective and single egg levels) without manual manipulation of the substrates as practiced in previous oviposition studies, which were lacking accuracy and precision. By using PDMS membrane-based through-hole micro-patterning, we studied fruit fly oviposition quantitatively in response to accurate and repeatable exposures to various agar surfaces.

In essence, pure PDMS platforms were found to be lethal to flies, yielding around 50% survival; however, a small 0.5mm diameter hole (0.016% agar exposure) was

capable of increasing the adult fly viability by approximately twofold. The viability in the assay was primarily affected by the amount of agar exposure and other parameters such as spacing between the access holes had no effect on sustaining 45 flies during the assay. With regards to oviposition, avoidance was exhibited on pure PDMS substrates. Acute jumps in the ovipositional rate was observed at a single $d=4\text{mm}$ (1% agar exposure) and seven $d=2\text{mm}$ (1.75% agar exposure) miniaturized through-holes, with both substrates producing agar-like responses. This showed that 1% exposure on an oviposition substrate area is adequate to produce agar-like responses. Furthermore, this miniaturization tool also provided us with a unique advantage to study oviposition site selection simply by comparing the number of eggs deposited inside the through-holes and the overall number of eggs laid on the entire substrate. We observed that by softening the agar, oviposition rate inside the through-hole increased and the attractiveness in site selection also increased- even in previously avoided though-holes (*e.g.* $d=0.5\text{mm}$). We also discovered that in order for site selection to occur, the substrate surface ought to have a repelling surface, an attracting surface, and a mechanical structure in place.

Knowing the fundamental effects of physical and chemical properties of the agar substrates on oviposition, in the second part of this thesis, we used the oviposition assay tool to develop chemical screening microfluidic chips. This was done ingeniously via using ice as a sacrificial layer. From this technique, two hybrid chips (*viz.* toxicity assay and multi-choice assay chip) were fabricated, and experiments were done using zinc and acetic acid to demonstrate the usefulness and the novelty of these devices.

The viability rates in both devices were ~100%, indicating good capabilities to sustain all adult flies within the 24h assay time period. Moreover, the oviposition rate in the zinc toxicity assay was found to be lowest for 70mM of zinc- thereby corroborating with current literature; while 0-20mM exuded similar attraction towards the oviposition since most of the eggs (>85%) were deposited inside the 4mm oviposition site. Avoidance in the 70mM zinc was also exhibited in the multi-choice assay; however, the oviposition site preferences were distinctly different between the attractants (*i.e.* 2mM and the control), which were not distinguishable using the toxicity chip. Similarly, in the acetic acid experiment, 0-5% acetic acid was shown to be attractive as ovipositional site, whereas the highest concentration, 15%, demonstrated a strong decline in oviposition rate, as in the multi-choice assay. Acetic acid at 5% was found to be more attractive than the control whereas the 15% was shown to be repugnant to the flies. In all the assays done, fewer eggs were found in comparison to the previous study [55], which used pure agar-PDMS substrates without the introduction of on chip dosing of chemicals. This phenomenon may be attributed to the extra time needed for learning and assessing the oviposition site choices due to more environmental factors.

5.2 Novelties of Research and Limitations

This is the first study to ever use micro-technology to devise a sensitive miniaturization tool for assaying oviposition behaviour and to innovate hybrid agar-PDMS microfluidic devices for chemical dosing and screening in free-flying adult

Drosophila. When compared to the conventional oviposition assay platforms on agar Petri dishes [35,36,45], the sensitive tool has provided miniaturization of oviposition sites. Many of the previous studies were done on a 60mm diameter petri dish [35,36]; therefore, we have significantly decreased the substrate area by 75 folds since the required oviposition site exposure found in our assay was a 4mm diameter through-hole. Additionally, this tool has demonstrated the ability for precise and repeatable patterning with PDMS for better quantification of the oviposition behaviour in response to the site exposure area, stiffness, and chemistry of the substrate in a microenvironment. The advancements of this sensitive miniaturization tool and its integration with a PDMS module to yield a dynamic dosing and chemical screening device has enhanced and allowed for in depth investigation of concentration dependency in adult fly oviposition, which has never been demonstrated in a microfluidic format. Furthermore, the microfluidic devices also provide accessibility to each site for chemical stimulation, which makes our platforms highly suitable for controllable and future dynamic high throughput screening assays.

In order to fabricate these microfluidic devices by integrating agar into designated areas within the PDMS module, we have developed a new method using ice sacrificial layer technique. This was done via loading water through the channel and freezing it to yield a sacrificial layer so that agar may be poured on the top. The novelty of this method

has advanced the microfabrication process for creating microfluidic devices for biological assays because water is a benign and known chemical that is tolerated by all organisms.

To date, a challenge that these two novel microfluidic devices faced was leakage between the PDMS and the agar. This was due to the extra pressure from the chemical infusion through the dosing apertures when loading the chemicals. The weak bond between the agar and PDMS interface tends to sometimes break causing chemical leakage and, thereby, flooding the whole oviposition platform. This flooding of the chip not only caused cross-talk of chemicals and the drowning of adult flies, it also shifted the patterned PDMS membrane. Therefore, further studies may be conducted to improve the bonding between PDMS and agar in order to produce a more robust device.

5.3 Future Applications

Upon addressing the aforementioned limitation and some iterations of this current model, our PDMS device can be used for a variety of assays. By solely using the sensitive oviposition quantification tool, automated egg collection, self-assembly and arraying devices can be achieved. And with the integration of these components with microfluidic layers (as in our two microfluidic devices), this technology may be further designed and developed for embryonic developmental studies. On the other hand, via future design iterations with our current agar-PDMS hybrid microfluidic device, automation of dynamic chemical dosing and screening may be easily done for the same applications using adult flies, as well as for other organisms and cell assays (Figure 21).

As shown in Figure 21, our improved dosing and screening devices would be placed on a platform with an adjustable camera that is connected to a computerized software, where the activities (*e.g.* the locomotion [22,23] or the viability [36]) of the flies may be recorded, and the oviposition rate can automatically be measured. This set-up with the two proposed devices may be used for further investigations of adult fruit fly viability and oviposition provided with different material substances loaded into each barrel simultaneously. In addition, they may also be used to answer more biological questions on the effects of different chemicals on oviposition and, specifically, in the field of assaying learning (decision-making [60,72]) and development [36] in adult *Drosophila melanogaster* and also in other fruit fly species (such as the *Ceratitis capitata*) and flying insects (such as moths). The results (*i.e.* oviposition and the viability rates) obtained in each of these oviposition site may then be compared using cross-correlation analysis.

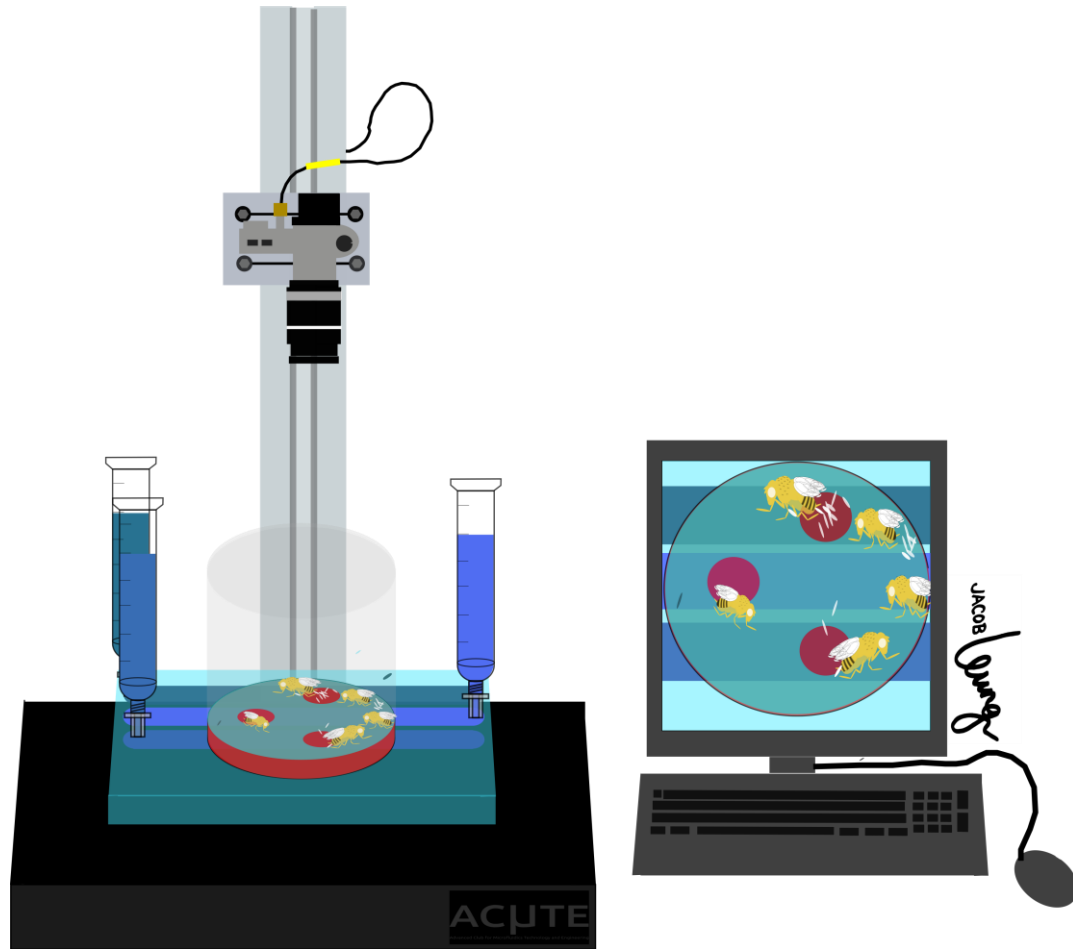


Figure 21: Automated experimental set-up of the multi-choice preference assay on adult *Drosophila melanogaster*. In this, the multi-choice preference assay chip will be prepared, as discussed in this thesis, and put onto a platform with an adjustable camera that is connected to the computerized recording and monitoring software. This would provide valuable information about the attractiveness of a substrate to the animal with time (*e.g.* by the average amount of time the flies stays on each substrate and the frequency of adult fly visit) that was not capable in our study without disturbing the fly environment.

In proposal, a specific route that this research may proudly embark upon is in one of the insect species that is in the same *Diptera* family as the *Drosophila melanogaster*

[73,74]. Currently, in Australia, flocks of sheep are experiencing infesting “flystrikes” due to the *Lucilia cuprina* (also commonly known as the Australian sheep blow-fly) [75,76]. These blow-flies are attracted to open flesh such as wounds and damp areas (*i.e.* the anus) of the sheep; thereby, causing maggot infested and exposed lesions that are disease-prone and life-threatening [76–78]. There are many ways of prevention [76,77,79]; however, aside from chemical and live organism techniques that are directed towards the adult stage flies, all of such prevention methodologies are only treatments and precautions taken after the flies’ oviposition. Currently, most chemical preventions only cause the eggs that were laid in the flesh to die or alter the neuronal signal of the fly causing them to only decrease in locomotion (thereby, harder for flies to move around from one place to another and reducing oviposition). Although these chemicals are somewhat effective, they have an utmost negative impact on the quality of the sheep’s wool (which is not preferred) [80–82]. Furthermore, another reported effective way is by using bacteria (*e.g. bacillus thuringiensis* [73,83]) as natural larvicides. This bacterium was recently studied and was shown to be toxic to only the larval stage of blow flies: thereby, claiming to stop their life cycle. Nevertheless, none has shown to cause avoidance of the blow fly towards the sheep itself, which is the root cause of the problem prior to their oviposition.

As a proposal, we believe that benign chemicals may be tested using our microfluidic chips for deterrence of the blowflies. Figure 22 shows and outlines the procedure that can be taken for this purpose. Firstly, various chemicals at different

corresponding chemical concentrations can be assayed using both the toxicity and the multi-choice assay devices (Figure 22a). From this, the behaviours caused by the chemicals may be assessed using the toxicity chip, indicating a binary result of either avoidance or preference towards the specific chemical. Subsequently, this chemical can be further investigated at different concentrations to check for the optimal effectiveness via the multi-choice preference assay chip. These deduced chemicals would be then experimentally sprayed on the real Australian sheep (Figure 22b) and the wool and the skin of the sheep will be examined for any traces of blow-flies or oviposition (Figure 22c). The chemical sprayed that was able to repel the flies would be the solution.

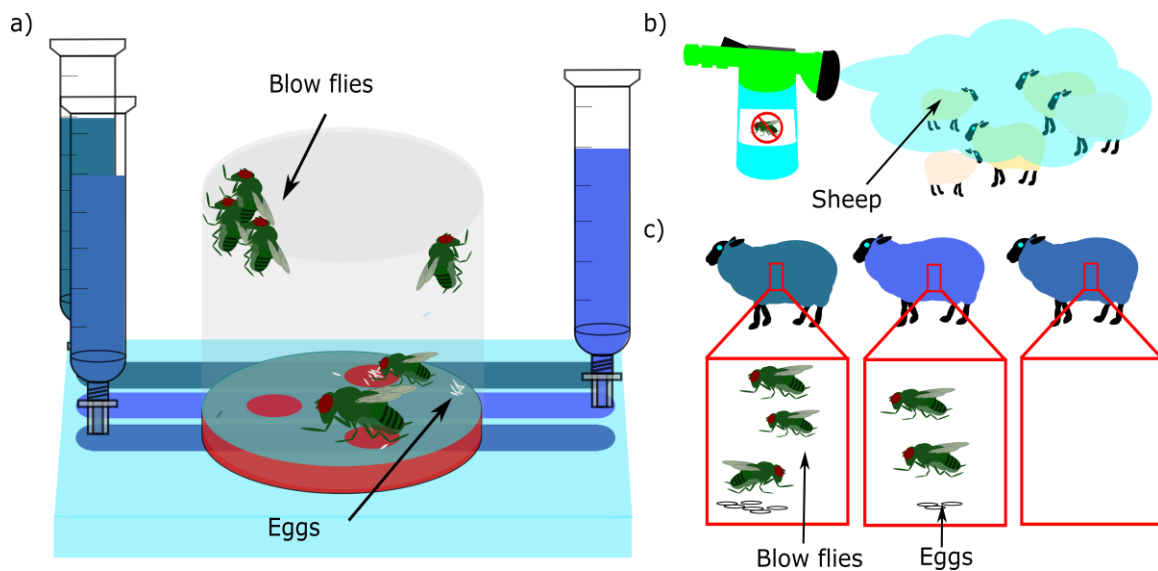


Figure 22: Proposed application of the microfluidic devices to be used on blow flies to prevent sheep fly-strike. In this experimental process, the blowflies will be used in lieu of the adult fruit flies in our assays. Different chemicals of different concentrations that are benign to sheep would be assayed using the toxicology and multi-choice device (a). Subsequently, the working chemical would be sprayed onto the wool of the sheep to cause avoidance to the adult blow flies (c). The wool and the embedded skin would be searched for both the blow flies and the eggs given different chemicals that would be investigated. Obviously, the sheep with the sprayed chemical that exudes no blow fly attraction and no oviposition would be desired.

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